carterra®

APPLICATION NOTE

Multi-Parameter Epitope-Centric Data Used to Elucidate Mechanisms of Action of Antibodies

Reveal mechanistically relevant information about your antibody library at the earliest stages of discovery with high throughput SPR epitope binning assay data merged with orthogonal data.

- **Maximize epitope diversity**
- **Build your IP portfolio**
- **Identify unique epitopes**
- **Discover mechanism of action**
- **Use binning and orthogonal data to help prioritize your research resources**

Introduction

Understanding the depth and breadth of an antibody panel's epitope coverage is important in assessing its quality and fitness for the intended purpose. In discovering therapeutic antibodies, it is highly desirable to identify clones with unique and functionally-relevant epitopes to build one's intellectual property portfolio. Knowing an antibody's mechanism of action is critical to its clinical success because it underpins safety and efficacy. An antibody's biological function is largely dictated by its binding specificity, as determined by its epitope, which can neither be predicted by *in silico* methods, nor shifted rationally by engineering, so must be selected empirically via screening at the earliest stages of research.

In this application note, we demonstrate how high throughput SPR facilitates the rapid assessment of an antibody library's epitopic diversity with minimal sample requirements. The one-on-many analyte-on-ligand format employed by high throughput SPR is uniquely suited to performing epitope binning assays on hundreds of antibodies per run. In this type of assay, all antibodies in a panel are tested for their ability to block one another's binding to their specific antigen in a pairwise and combinatorial manner. This enables a "blocking footprint" to be assigned per antibody, and antibodies sharing similar footprints are clustered together in a family or "bin." When performed in high throughput, the bin members are likely to share highly similar or near-identical binding modes. The example described herein reinforces this point, and is supported by structural and mutagenesis studies, demonstrating the exquisite epitope resolution that can be achieved by high throughput epitope binning assays, informing decision-making in drug discovery.

Method

A panel of 70 sequence-unique monoclonal antibodies were supplied as purified IgG and arrayed onto a G-COOH coated chip (Ssens bv, NL) by standard amine-coupling using two consecutive prints of a 48-channel continuous flow microspotter (CFM), by Carterra Inc (formerly Wasatch Microfluidics), as follows. The CFM was primed with run buffer (PBS + 0.01% Tween20). The printhead was docked onto the first print location (at the top half of the chip) and spots were activated by cycling freshly prepared solution of 150 µl 0.4M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) + 150 µl 0.1M N-hydroxysulfosuccinimide (SNHS) in 5 ml 0.1M MES pH4.5 (coupling buffer) for 7 min and then 48 antibodies, each diluted to 10 µg/ml in coupling buffer, were cycled for 15 min.

The spots were rinsed with run buffer and the print routine was repeated at the second print location (at the bottom half of the chip) to couple the remaining antibodies. The printed chip was docked into an MX96 SPR imager (IBIS Technologies, NL) and quenched with a 7-min injection of 1 M ethanolamine pH 8.5. The MX96 was primed with run buffer and an epitope binning experiment was performed in a "classical sandwich" assay format where each binding cycle comprised 3 steps: (1) a 3-min injection of antigen (30 nM IsdB) followed immediately by (2) a 5-min injection of antibody (10 µg/ml), and (3) a 30-sec injection of 75 mM phosphoric acid to regenerate the surfaces.

Each antibody was used in the role of both ligand (immobilized via the CFM) and analyte (injected in the MX96).

A single unattended epitope binning experiment took 30 hours to run and required only 5 µg per antibody to address a comprehensive pairwise binning matrix in a 96 x 96 "analyte x ligand" format. Data were analyzed in Carterra's Epitope Tool to generate the sorted heat map, networks, dendrograms, and communities. The data were groomed to remove poorly behaving analytes and ligands, resulting in a final heat map distilled to 66 analytes and 54 ligands, representing 70 unique antibodies.

Results

High Throughput SPR was used to assess the epitope coverage of a panel of 70 sequence-unique monoclonal antibodies sourced from single-cell cloning of memory B cells in blood samples collected from healthy donors that were reactive to iron-regulated surface determinant B (IsdB), a protein involved in iron acquisition of *Staphylococcus aureus*. IsdB is a commensal pathogen, so understanding the host-microbe relationship provides insight to rationalizing the likelihood, type, and severity of disease.

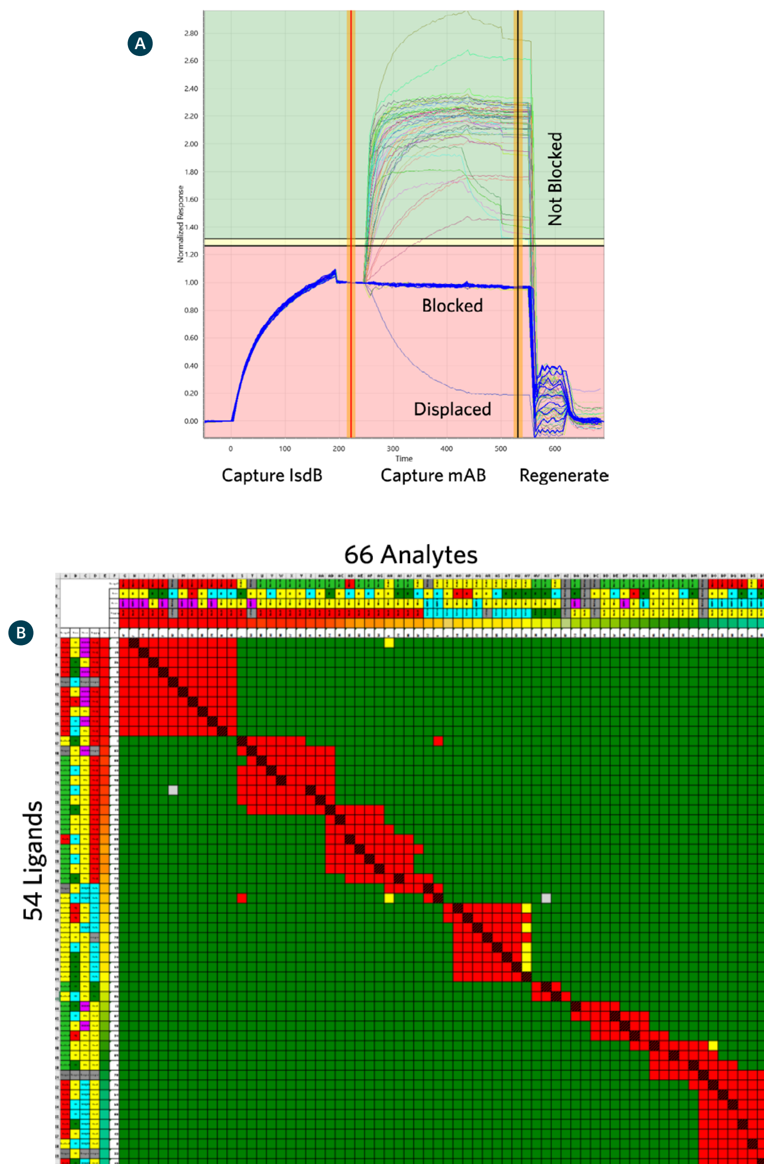

Figure 1: Results from an epitope binning experiment on a panel of 70 unique-sequence mAbs. (A) Sensorgram overlay on a single mAb-coated spot and (B) Heat map with orthogonal data added to column and row headers.

The epitope binning experiment was performed in a classical sandwich assay format[1], using only 5 µg per antibody, and running unattended for 30 h. The data was analyzed quickly and easily in Carterra's Epitope Tool, which provides various options for viewing that data while maintaining connection to the raw data at every step. **Figure 1A** shows an example of the sensorgrams obtained in multiple binding cycles on a single antibody-coated spot. The data are normalized at the end of the antigen capture step and a threshold setting is applied, relative to a buffer blank "analyte" injection (blue curves). Curves falling above this line indicate analytes that can sandwich pair with the immobilized antibody (ligand), whereas analytes that give no signal (resembling the buffer blanks) are blocked. Thus, it is inferred that two antibodies that can form a sandwiching pair bind non-overlapping epitopes on their specific antigen whereas two antibodies that block one another target overlapping epitopes.

A unique advantage of the classical sandwich assay format is that it reveals nuanced behaviors too, such as antibody displacement, as characterized by inverted sandwiching responses, falling well below the buffer blank threshold setting, suggesting that the ligand and analyte cannot coexist stably on their specific antigen owing to their closely adjacent or minimally overlapping epitopes on their target antigen[2]. This affords an even more granular epitope discrimination since displacement is a hybrid mechanism between antibody sandwiching and blockade. When the analysis is expanded to the entire data set, a heat map is constructed for each analyte/ligand pair, showing the sandwiching and blocking pairs as green or red cells respectively (**Figure 1B**). For simplicity, the displaced pairs are treated as "blocked".

Carterra's proprietary network plotting software provides an intuitive way of navigating the binning results. In a network plot, the nodes represent the antibodies (identified by number), the chords indicate the pairwise blocking relationships, and the envelopes inscribe the bins. Further, by merging orthogonal data, the networks can be colored in different ways, thereby facilitating the organization of multi-parameter data, while keeping an epitope-centric focus. In this study, the 70 unique antibodies fell into several distinct bin clusters containing two subsets of neutralizing clones (**Figure 2A**), which were populated by multiple donors (**Figure 2B**).

Interestingly, the two neutralizing bins showed a heavily biased use of two immunoglobulin heavy chain germlines, namely IGHV4-39 (bin C) and IGHV1-69 (bin P) (**Figure 2C**). Mutagenesis studies showed that these distinct bins corresponded to neutralization of different IsdB subdomains, namely NEAT1 and NEAT2 respectively (**Figure 2D**). To elucidate the binding mechanism of NEAT2 and the IGHV1-69-derived neutralizing antibodies (bin P), crystal structures of two Fabs from different donors in complex with NEAT2 were determined and found to share near-identical binding modes[3].

Mutagenesis studies showed that the other IGHV1-69-derived antibodies in bin P bound IsdB in a similar manner, since a single mutation at the surface-exposed F54 position (F54A) resulted in >100-fold loss in affinity for all bin P antibodies. Similarly, by combining structural and mutational data they showed that all antibodies in the IGHV4-39-derived NEAT1-neutralizing subset (bin C) interact with NEAT1 in a similar fashion, suggesting that the two bin clusters resolved different mechanisms of neutralization, reinforcing the significance of the binning data.

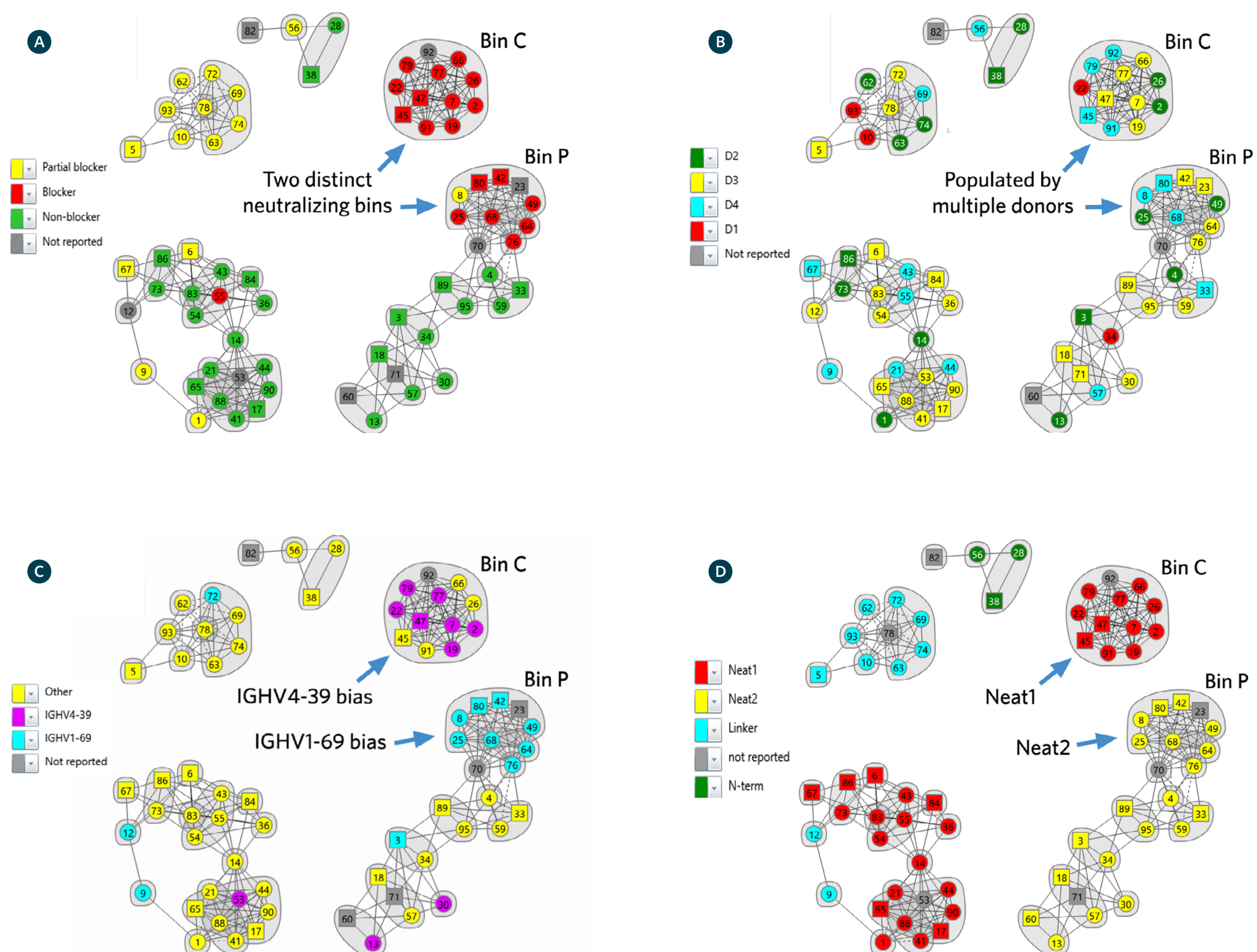

Figure 2: Network plots showing bin clusters colored by orthogonal data; (A) Neutralization, (B) Donor, (C) Germline, and (D) Mutagenesis-based Mapping

Another way of viewing binning data in Carterra's Epitope software is in terms of a binning dendrogram (**Figure 3A**) and its corresponding community plot (**Figure 3B**). Here, we show an example using a custom cut-height, giving ten groups or "communities" that is a coarser analysis than the networks shown above, but clearly demarcates the two neutralizing bins, C (orange) and P (red).

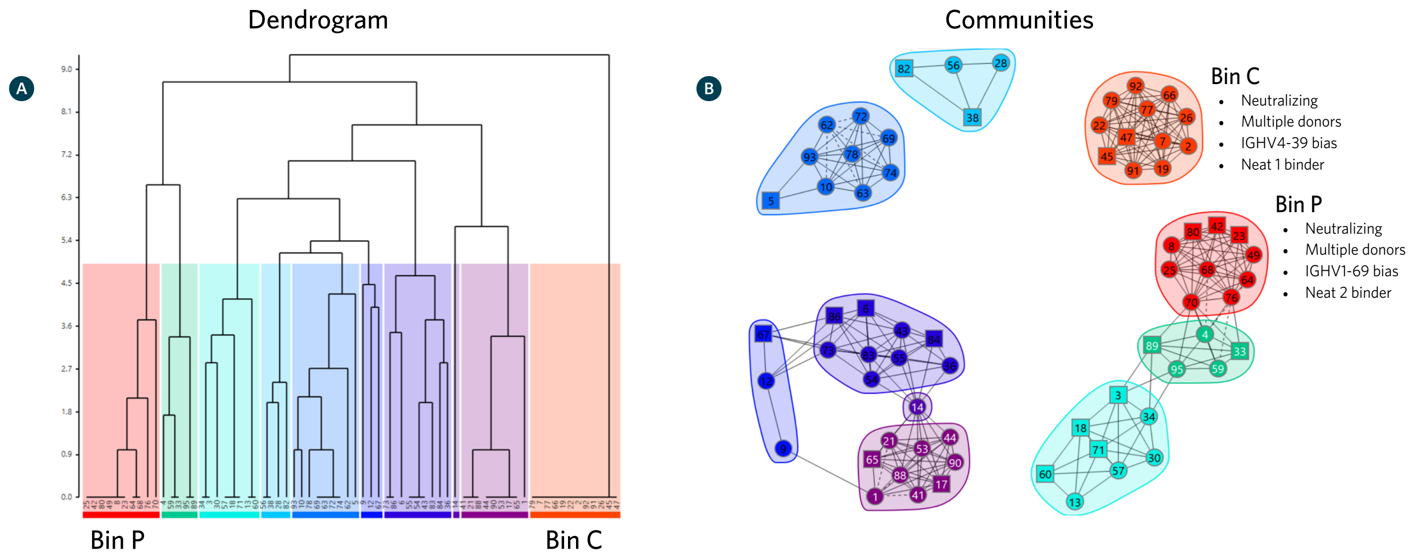

Figure 3. Alternate analysis showing (A) custom-cut dendrogram and (B) corresponding communities. The orthogonal data for the germline-encoded neutralizing bins are summarized.

Summary

This example shows how the results from a single high throughput SPR epitope binning experiment on a panel of 70 sequence-unique antibodies revealed two distinct bin clusters that correlated with germline-encoded neutralization of a Staphylococcus aureus virulence factor by the human antibody repertoire. Due to the simplicity, speed, and minimal sample requirement of the binning assay, blocking relationships were established on the whole panel of antibodies at the early stages of discovery, allowing sequence-epitope relationships to be identified and probed further with more focused lower-throughput and resource-intensive methods. The functional and biological significance of the sequence-epitope relationships was realized upon merging cell-based hemoglobin-blocking (neutralization) data. Combining the use of both structural and mutagenesis techniques showed that the two distinct bin clusters neutralized IsdB by different MOAs, with bin members showing near-identical binding modes. The binning data was critical in guiding these studies that led to an exquisite demonstration of how certain germlines, while part of the adaptive immune system, may have evolved under selective pressure to converge upon an encoded binding motif innately capable of neutralizing, via different MOAs, a structurally conserved protein domain involved in pathogen iron acquisition.

While the binning data described here was acquired on a 96-ligand array, recent advances by Carterra have expanded the capacity of these assays to 384-ligand arrays[4], enabling characterization of larger antibody libraries at the earliest stages of discovery.

References

1. Abdiche, YN, et al. Exploring blocking assays using Octet, ProteOn, and Biacore biosensors. Anal. Biochem. 386, 172–180 (2009).
2. Abdiche, YN, et al. Antibodies targeting closely adjacent or minimally overlapping epitopes can displace one another. PLoS One. 12, 1–22 (2017).
3. Abdiche, YN, et al. Germline-encoded neutralization of a Staphylococcus aureus virulence factor by the human antibody repertoire. Nat. Commun. 7, 13376 (2016).
4. Sivasubramanian A, et al. Broad epitope coverage of a human in vitro antibody library. mAbs, DOI:10.1080/19420862.2016.1246096 (2016).

Carterra technology is protected by the following patents and other patents pending:
8,210,119, 8,211,382, 8,383,059, 8,999,726, 9,682,372, 9,682,396

© 2019 Carterra Inc. Carterra, the Carterra Logo and all other trademarks are property of Carterra unless otherwise specified.

carterra®

info@carterra-bio.com

825 N 300 W Suite C309
Salt Lake City, UT 84103

www.carterra-bio.com