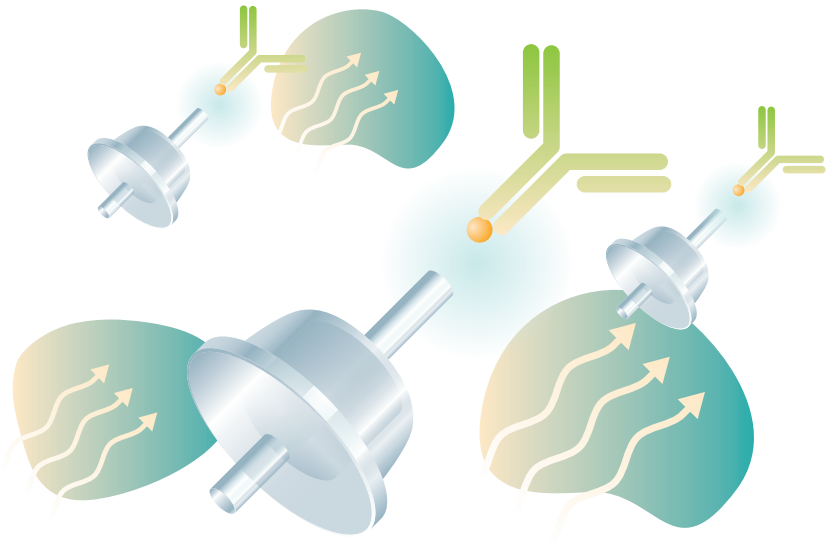

Automated 32x32 Epitope Binning in a Single Assay

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Introduction

The selection of lead candidates in the development of therapeutic monoclonal antibodies (mAbs) is a critical decision. Designing a mAb that exhibits all the desired biological and pharmaceutical properties is challenging, and therefore, this process is primarily empirical. Numerous analytical studies are performed to identify leads with desirable characteristics, and among them, the evaluation of binding affinity is considered a significant factor. However, relying solely on affinity to select antibodies can be problematic since the highest affinity mAbs may not necessarily exhibit optimal activity. Therefore, it is important to consider additional factors such as specificity, selectivity, and biological relevance to ensure the selection of the most promising lead candidates for further development.

The classification of monoclonal antibodies (mAbs) based on their binding behavior can be a valuable tool for selecting promising candidates for further development. Such classification often involves grouping mAbs with similar epitope binding regions, which are known to share similar biological functions. These groups or "bins" can streamline the candidate screening and selection process by providing a pool of similarly behaving mAbs for further investigation. Epitope binning studies are commonly used to classify mAbs into these groups and involve pairwise testing of all mAbs against each other. A classical epitope binning assay typically involves first binding an antigen with the desired epitope to a mAb candidate. Then, a second mAb is added, and the results are interpreted based on whether binding occurs or not. If binding occurs, it is assumed that the second mAb targets a different epitope. Conversely, if binding is blocked, it suggests that the binding epitopes are the same or closely related. Overall, epitope binning studies can provide useful information for selecting and characterizing mAbs for therapeutic applications.

Gator® Pro System

The Gator® Pro system is a highly advanced analytical instrument specifically designed for label-free, real-time measurement of biological molecules using biolayer interferometry (BLI). The system's versatility allows for a wide range of applications, including but not limited to titer determination, quantitation of unknown samples, ranking of antibodies by off-rate, measurement of binding affinity, interrogation of target antigen binding, antibody affinity maturation, and epitope binning. These assays can be performed rapidly and accurately, offering unprecedented levels of precision and throughput for biological assays.

The Gator® Pro system is the fastest epitope binning tool available on the market today, capable of performing 32 x 32 epitope binning in just 8 hours, utilizing a single automated tandem or classical sandwich assay. This offers a significant advantage over other BLI assays and traditional methods, allowing for high-throughput epitope binning with unprecedented speed and accuracy.

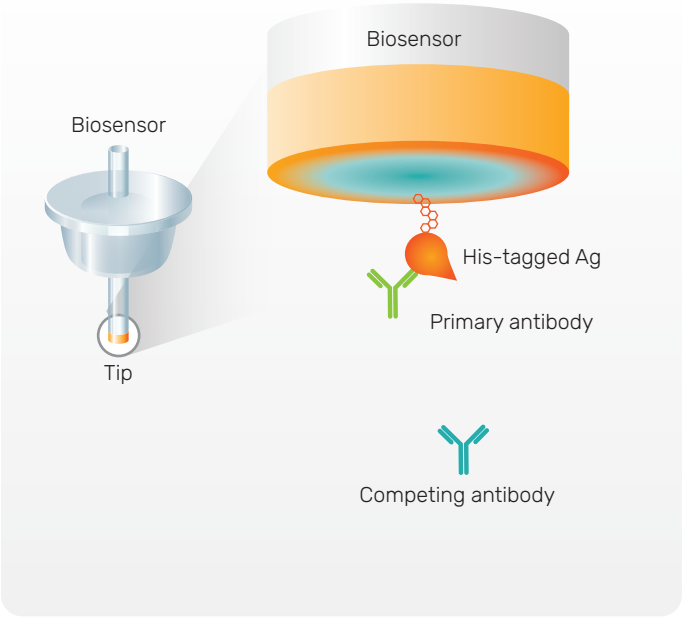
In a recent study, 32 antibodies were subjected to epitope binning analysis using the Gator® Pro system. These antibodies were initially binned into 5 groups using classical ELISA techniques that took over 24 hours. In addition to the 32 x 32 binning assay for initial categorization, subsequent 24 x 24 and 8 x 8 assays were performed to further classify a selection of antibodies into sub-groups. These results demonstrate the efficacy and versatility of the Gator® Pro system for high-throughput analysis of biological molecules, especially in the context of epitope binning, which is crucial for selecting and characterizing therapeutic monoclonal antibodies.

Monoclonal Antibodies for SARS CoV-2

Monoclonal antibodies generated against the spike protein of the SARS CoV-2 virus are proven effective therapies in the ongoing fight against the COVID 19 disease. During the early days of the COVID 19 pandemic, Curia rapidly generated recombinant SARS-CoV-2 spike protein, performed immunizations using its immunologically diverse PentaMice® platform, and utilized an optimized hybridoma-based discovery workflow to generate a broad panel of spike-binding mAbs. As shown in Figure 1, 32 antibodies from this initial discovery campaign were grouped into 5 different categories based upon their different binding domains and had previously been binned using classical sandwich ELISA assays.

No.	mAb ID	Binding domain
1	mAb1	RBD
2	mAb2	RBD
3	mAb3	RBD
4	mAb4	RBD
5	mAb5	RBD
6	mAb6	RBD
7	mAb7	RBD
8	mAb8	RBD
9	mAb9	RBD
10	mAb10	RBD
11	mAb11	RBD
12	mAb12	RBD
13	mAb13	RBD
14	mAb14	RBD
15	mAb15	RBD
16	mAb16	S1 non-RBD
17	mAb17	S1 non-RBD
18	mAb18	S1 non-RBD
19	mAb19	S1 non-RBD
20	mAb20	S1 non-RBD
21	mAb21	S2
22	mAb22	S2
23	mAb23	S2
24	mAb24	S2
25	mAb25	S2
26	mAb26	S2
27	mAb27	S2
28	mAb28	S2
29	mAb29	non-RBD, non-S1, non-S2
30	mAb30	non-RBD, non-S1, non-S2
31	mAb31	non-RBD, non-S1, non-S2
32	mAb32	non-RBD, non-S1, non-S2

Figure 1: Monoclonal antibodies for epitope binning study. The mAbs for epitope binning were divided into groups based upon previous binning assays using classical ELISA techniques.



32x32 assay statistics	
32	Samples
32	Assays
32	HIS probes
3	384-well probes
7	Hours

Figure 2: Tandem 32x32 Epitope Binning Assay on the Gator Pro System. The HIS probe, which recognizes His-tagged proteins, is added to a solution containing the His-tagged antigen, (His-tagged Spike protein or His-tagged S2 region of Spike protein). After equilibration the probe is added to a solution containing the primary antibody. The probe is then added to a solution containing the competing antibody.

Assay Design

Figure 2 shows a schematic of the assay workflow for tandem 32x32 epitope binning. For these studies Gator® Anti-His (HIS) probes were used (Part No. 160009). These probes are precoated with a high-affinity, monoclonal antibody that captures polyhistidine-tagged (His-tagged) proteins.

The initial 32x32 binning assay used His-tagged Spike protein as the antigen (SARS-CoV-2 S protein, His Tag, ACRO Biosystems, Catalog# SPN-C52H9). HIS Probes immobilize the His-tagged antigen to the sensor surface. After equilibration, probe capture the primary antibody from sample well. The last step is to react to the competing antibody.

Figure 3 showcases representative sensorgrams for three types of assay results: blocking, non-blocking, and undecided. These assay results are used to determine whether the monoclonal antibodies (mAbs) target identical or different epitopes. In the blocking assay (indicated by red), the primary antibody saturates the binding site, and if no change is detected in the sensorgram after adding the second antibody, it suggests that the binding site is blocked. This indicates that the two mAbs recognize identical or highly similar epitopes. On the other hand, in the non-blocking assay (indicated by green), the addition of the second antibody produces a change in the sensorgram, which is presumed to be non-blocking antibodies with different binding sites. In the undecided assay (indicated by yellow), little or no binding signal is observed from the primary antibody. This is most likely due to a low functional concentration, making it difficult to discern the relationship between the two antibodies.

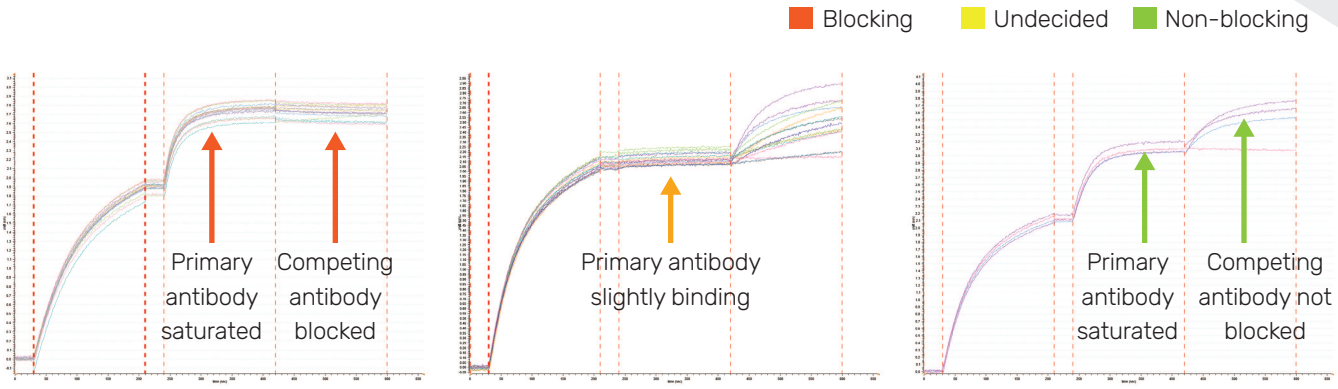


Figure 3. Representative sensorgrams for binning assay. Blocking antibodies (Red – blocking) are shown on the left and recognize identical or very similar epitopes. Non-blocking antibodies (Green – non-blocking) are shown on the right and recognize different epitopes. Inconclusive results (Yellow – undecided) are shown in the middle and can occur when low or no binding is observed for the primary antibody.

32x32 Epitope Binning

Using the 32x32 epitope binning assay, 13 mAbs were classified into five distinct groups. Grouping analysis revealed that mAbs 2, 3, and 4 belonged to a single group, while mAbs 5, 6, and 8 were assigned to a different group. Additionally, mAbs 7, 17, and 27 were classified into individual groups (Figure 4). Further analysis showed that mAbs 5, 6, and 8 blocked each other, but not other mAbs, whereas mAbs 17, 27, and 7 only blocked themselves, with each forming a separate group.

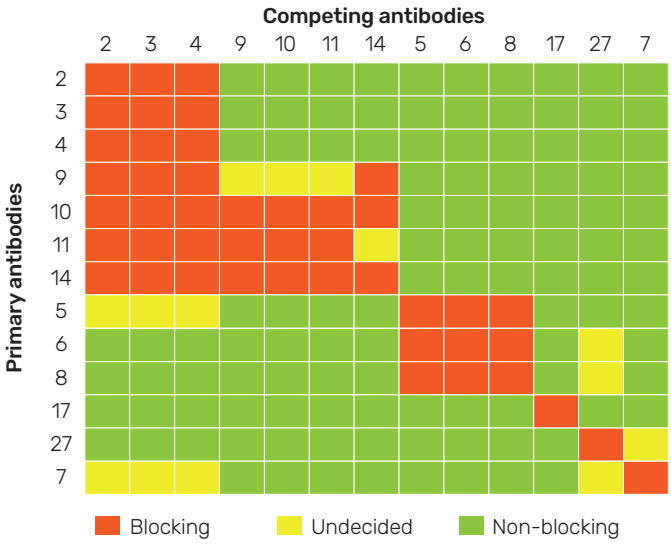


Figure 4. 32x32 binning assay results. Five major groups were observed from the 32x32 binning assay: (1) 2,3,4; (2) 5,6,8; (3) 7; (4) 17; (5) 27. Subgroups were also observed for mAbs 9,10,11,14. Different colors represent different groups.

No.	mAb ID	Binning domain
1	mAb1	RBD
2	mAb2	RBD
3	mAb3	RBD
4	mAb4	RBD
5	mAb5	RBD
6	mAb6	RBD
7	mAb7	RBD
8	mAb8	RBD
9	mAb9	RBD
10	mAb10	RBD
11	mAb11	RBD
12	mAb12	RBD
13	mAb13	RBD
14	mAb14	RBD
15		Negative Control
16	mAb16	S1 non-RBD
17	mAb17	S1 non-RBD
18	mAb18	S1 non-RBD
19	mAb19	S1 non-RBD
20	mAb20	S1 non-RBD
21	mAb21	S2
22	mAb22	S2
23	mAb23	S2
24	mAb24	S2
25	mAb25	S2
26	mAb26	S2
27	mAb27	S2
28	mAb28	S2
29	mAb29	non-RBD, non-S1, non-S2
30	mAb30	non-RBD, non-S1, non-S2
31	mAb31	non-RBD, non-S1, non-S2
32	mAb32	non-RBD, non-S1, non-S2

The group containing 2, 3, and 4 not only block themselves but also have blocking abilities against subgroups containing 9, 10, 11, and 14. As shown in Figure 5, left, blocking is observed when the primary mAb (row) is 9, 10, 11, or 14 against a secondary mAb (column) of 2, 3, or 4. However non-blocking is observed when the mAbs are reversed. This may be due to differences in binding rates and differences in the lengths of amino acid residues to bind. For example, when mAb3 is the primary antibody it most likely binds slowly or with less residues allowing mAb9 to bind (Figure 5, center). Whereas when mAb9 is the primary antibody, it binds faster, or to more residues, creating steric hinderance and preventing mAb3 from binding (Figure 5, right).

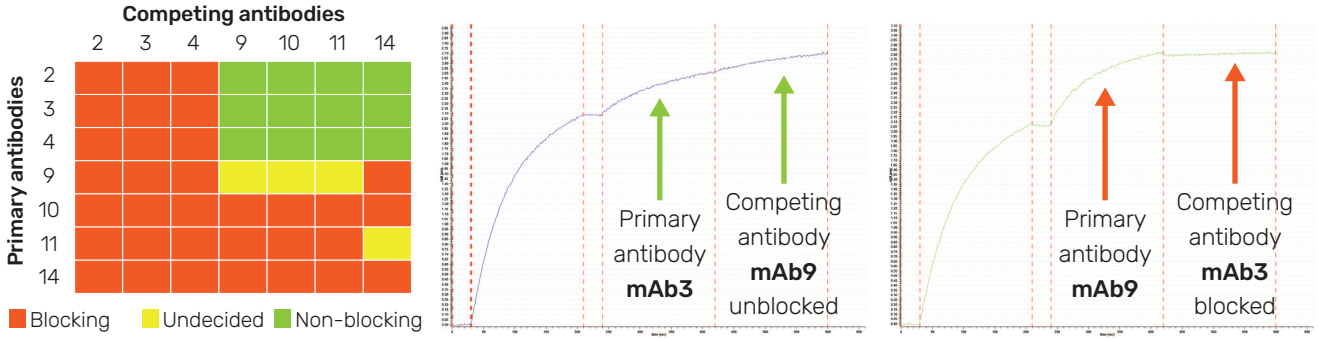
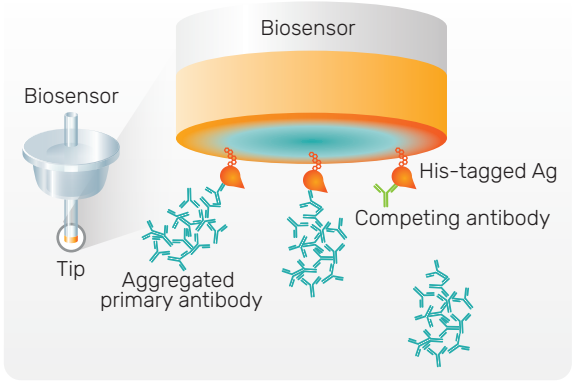


Figure 5. mAb subgroup detail. The sensorgram shows that mAb3 does not block mAb9 when it is used as the primary antibody. However, when mAb9 is used as the primary antibody it does block mAb3 from binding.

Protein Aggregation

From the 32x32 binning assay 13 antibodies gave conclusive information with 5 major groups with subgroups. However, more than half (19 mAbs) did not bind and were inconclusive. This could be due to several reasons including protein aggregation, insufficient concentration, or a binding epitope that is within less accessible regions when the Spike protein is bound through a His-tag.

As shown in Figure 6, with protein aggregation a high binding signal is observed but the surface is not saturated. The shift in binding is similar to the binding of a primary antibody but the binding curve never plateaus. Instead, continuous binding is observed suggesting that many bindings sites of the primary antibody are still available. When a competing antibody that shares the same binding site is added, the curve continues to increase even though blocking should be observed.



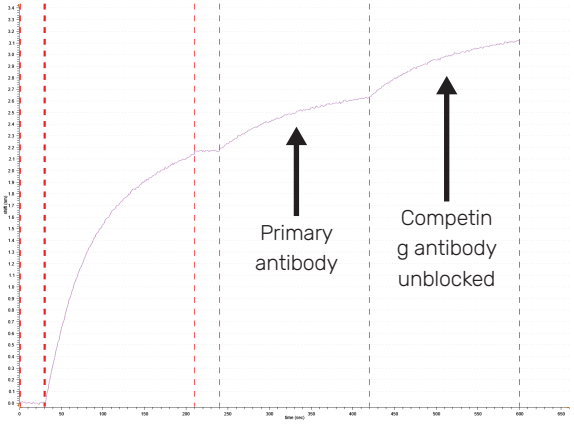


Figure 6. Identifying protein aggregation. Binding occurs with the primary antibody but continues to rise and never plateaus and equilibrates. After the addition of a competing antibody, the binding curve should plateau and show no upward shift. However, a continuous rise indicates protein aggregation is most likely occurring.

24x24 Epitope Binning

Given that more than half of the inconclusive antibodies appear to target the S2 region of the Spike protein, we conducted a 24x24 epitope binning experiment using only the S2 region (His-tagged) as the antigen at a concentration of 10 µg/mL. Specifically, we utilized the SARS-CoV-2 Spike S2 Protein, His Tag, which is available from ACRO Biosystems (Catalog# S2N-C52H5). In this experiment, 24 primary antibody samples were tested at a concentration of 15 µg/mL against 24 competing antibodies at a concentration of 5 µg/mL.

Upon analyzing the 24x24 matrix, we were able to identify three distinct groups that bind specifically to the S2 unit. Antibody 22 was found to form one of these groups, while mAbs 23, 24, and 25 were observed to form a second group. A third group was identified with mAb 26. While it was anticipated that mAb 27 would also exhibit binding based on its binding domain, the results were inconclusive, as were those for the remaining antibodies in the matrix. There are several possible reasons for these inconclusive results, including insufficient concentration, inadequate association time, or binding to a different and inaccessible region of the Spike protein.

No.	mAb ID	Binning domain
16	mAb16	S1 non-RBD
17	mAb17	S1 non-RBD
18	mAb18	S1 non-RBD
19	mAb19	S1 non-RBD
20	mAb20	S1 non-RBD
21	mAb21	S2
22	mAb22	S2
23	mAb23	S2
24	mAb24	S2
25	mAb25	S2
26	mAb26	S2
27	mAb27	S2
28	mAb28	S2
29	mAb29	non-RBD, non-S1, non-S2
30	mAb30	non-RBD, non-S1, non-S2
31	mAb31	non-RBD, non-S1, non-S2
32	mAb32	non-RBD, non-S1, non-S2

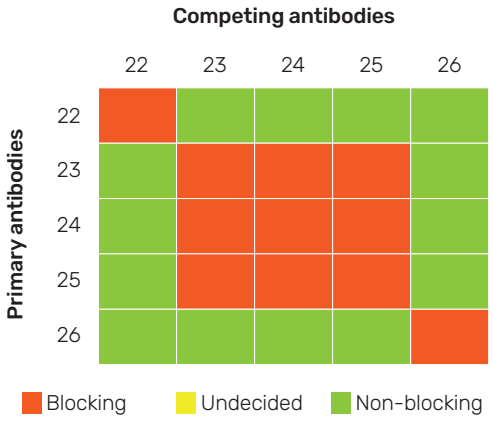


Figure 7. 24x24 binning assay results. Three major groups were observed from the 24x24 binning assay: (1) 23,24,25; (2) 22; (3) 26.

8x8 Epitope Binning

Subsequently, we performed a follow-up 8x8 matrix experiment using the same His-tagged S2 protein as the antigen and at the same concentration as in the 24x24 matrix (10 µg/mL). However, in this experiment, we tested both the primary antibody and the competing antibody at double the concentrations used previously (30 µg/mL and 10 µg/mL, respectively). Moreover, we increased the association time to 10 minutes in order to facilitate binding of the primary antibody.

No.	mAb ID	Binning domain
1	mAb1	RBD
4	mAb4	RBD
21	mAb21	S2
22	mAb22	S2
23	mAb23	S2
24	mAb24	S2
25	mAb25	S2
26	mAb26	S2

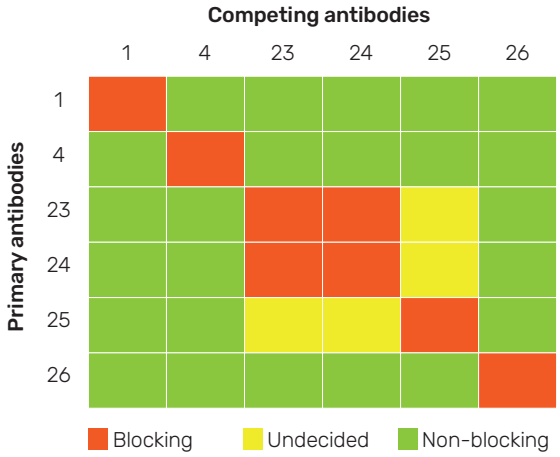


Figure 8. 8x8 binning assay results. Four major groups were observed from the 8x8 binning assay: (1) 1; (2) 4; (3) 23,24,25; (4) 26.

With the increased concentrations and association times, the 8x8 binning experiments provided more conclusive results. Specifically, we were able to identify six out of eight antibodies that exhibited binding to the S2 region of the Spike protein. The data revealed four major groups with subgroups, as shown in Figure 8. Notably, two of the groups identified matched those previously observed to bind the S2 unit in the 24x24 matrix, with mAbs 23, 24, and 25 forming one group and mAb 26 in another group. Moreover, some previously inconclusive mAbs could now be grouped with mAb 1 in its own group, while mAb 4 was classified into a separate group.

Figure 9. Groups identified from all assays. Nine groups were identified from all three assays (32x32, 24x24, and 8x8). Different colors represent different groups.

No.	mAb ID	Binding domain
1	mAb1	RBD
2	mAb2	RBD
3	mAb3	RBD
4	mAb4	RBD
5	mAb5	RBD
6	mAb6	RBD
7	mAb7	RBD
8	mAb8	RBD
9	mAb9	RBD
10	mAb10	RBD
11	mAb11	RBD
12	mAb12	RBD
13	mAb13	RBD
14	mAb14	RBD
15		Negative Control
16	mAb16	S1 non-RBD
17	mAb17	S1 non-RBD
18	mAb18	S1 non-RBD
19	mAb19	S1 non-RBD
20	mAb20	S1 non-RBD
21	mAb21	S2
22	mAb22	S2
23	mAb23	S2
24	mAb24	S2
25	mAb25	S2
26	mAb26	S2
27	mAb27	S2
28	mAb28	S2
29	mAb29	non-RBD, non-S1, non-S2
30	mAb30	non-RBD, non-S1, non-S2
31	mAb31	non-RBD, non-S1, non-S2
32	mAb32	non-RBD, non-S1, non-S2

Summary

From the three binning experiments, 19 antibodies binned into 9 different groups (Figure 9). The remaining 13 antibodies remained inconclusive, with low functional concentration likely being a main factor. As a follow-on BLI experiment the actual antibody functional concentrations should be evaluated prior to further studies. Once accurate concentrations are determined, the 32x32 matrix can be re-run using the whole Spike protein, in addition to the use of other Spike protein variants or other binning matrices.

Conclusion

Epitope binning is a process used to group monoclonal antibodies based on their ability to bind to distinct or overlapping epitopes on a specific antigen. In this study, we used the high-throughput Gator® Pro epitope binning to analyze a panel of mAbs against the SARS-CoV-2 spike protein. The results showed that the mAbs could be grouped into distinct bins based on their binding patterns, which allowed for the identification of mAb combinations that could potentially have synergistic effects.

It's worth noting that these mAbs were all screened and selected based on ELISA, which may not always be the optimal assay for identifying mAbs that work well in other assays such as BLI, FACS, or Western blotting. Therefore, it's crucial to identify a large panel of mAbs in any antibody discovery campaign, which can then be used to assess and define epitope coverage using effective binning approaches like Gator® system.

Furthermore, the binning data could be useful in designing a diagnostic lateral flow assay, where pairs of mAbs that bind to different spike domains can be identified. Targeting the S2 domain for this purpose could be a novel approach, especially in patients treated with neutralizing therapeutic mAbs, which are typically RBD binders. Overall, these findings highlight the potential of epitope binning in identifying mAb combinations with synergistic effects and designing diagnostic assays for SARS-CoV-2.

Lastly, epitope binning on Gator® Pro can identify major groups and subgroups within large pools of antibodies. Aggregation of antibodies can also be detected. An important consideration is to accurately evaluate the functional concentration of the antibody prior to any binning experiment. With fast and easy assay development, the Gator® Pro system can run multiple assays using different antigens and samples including 32x32, 24x24, 8x8 matrices to better determine different binning groups in the antibody pool.

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