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APPLICATION NOTE

Reveal the Epitope Diversity of Your Antibody Library Using High Throughput SPR: A Case Study Using Progranulin

Highlights

- **Carterra's LSA facilitates pairwise competition or epitope binning assays on hundreds of monoclonal antibodies (mAbs) in parallel to reveal the epitope landscape of your antibody library.**
- **Knowing your epitope coverage is key to the selection of mAbs with unique mechanisms of action and to securing intellectual property.**
- **Binning (competition assays) and mapping (screening against antigen variants, in this case, chimeras) can be performed in the same assay, streamlining epitope characterization and providing complementary information.**
- **Sample consumption is exceptionally low; typically, only 5 µg per mAb is required to generate a full 384 x 384 competition matrix, and doesn't scale with the size of the antibody panel, providing an extremely efficient way of using precious material.**
- **Merging binning data with orthogonal data, such as mapping, library source, and cross-reaction to orthologs or homologs of the target antigen, provides a more comprehensive analysis of your antibody library, which facilitates the identification of clones with uniquely desirable characteristics.**

Introduction

High throughput epitope binning provides an efficient method for interrogating the epitope diversity of a panel of monoclonal antibodies (mAbs). Typically, binning experiments are limited in scope and complexity due to the geometric way the assays scale on traditional interaction analysis platforms. For example, a 10 x 10 set of antibodies requires 100 unique tests to complete a bi-directional competition map, whereas a 96 x 96 set would require nearly 10,000 individual analyses[1].

Carterra's LSA is a high throughput SPR platform that enables the interaction analysis of up to a 384 x 384 antibody matrix in a single unattended run without the use of additional robotic integration or large numbers of sample plates. Performing epitope binning assays on a large panel of mAbs (up to 384 unique clones at full capacity) is advantageous because the competition picture gains resolution, detail, and nuance with the size of the antibody panel under investigation. Very little sample is required, making these assays particularly amenable to early-stage research where antibodies are numerous, but available only in low quantities.

The Model Panel

The Antibodies

Carterra has assembled a panel of 29 anti-human progranulin (hPGRN) mAbs for use as a model set of reagents to demonstrate the efficient use of high throughput SPR in performing epitope binning assays. Using the classical sandwich assay format[1] we show that a binning experiment on an array of 384 mAbs is simple to set up and easy to analyze in our industry-leading analysis software that provides intuitive visualization tools to speed up data interpretation. This antibody set also provides examples of additional interaction profiles beyond simple competition, including clones with closely adjacent or minimally overlapping epitopes that kick-off their neighbor[2], as well as asymmetrical competition profiles due to allosteric effects[3].

The Target

Progranulin (PGRN) is a 593-amino acid glycoprotein with a molecular weight (MW) of 62.5 kDa. It consists of seven cysteine-rich granulin domains, A-G, and a truncated paragranulin domain referred to here as p[4]. The multi-domain architecture of PGRN, therefore, lends itself to mapping because bins can be mapped to specific subdomains,

which can be used to assess the epitope coverage of a given antibody library and benchmark it to other libraries against the same target.

The Chimeras

To map the mAbs to specific subdomains we used a panel of five recombinant mouse/human chimeras comprised of the mouse PGRN sequence with two or three of the granulin domains swapped out for their human counterpart[5]. This chimeric swap strategy allowed the human-specific mAbs to be mapped to a granulin subdomain (A, B, CD, E, F, G, or p). Clones that were human+mouse cross-reactive were not amenable to epitope mapping by the chimeric swap strategy, so their assignments were inferred based upon their cross-blocking of the human-specific mAbs with known assignments. In this way we were able to combine mapping and binning to assign subdomains to all the mAbs in the panel, providing a rich data set. The mapping and binning were performed in a single experiment by including the antigens in the immobilized array, taking advantage of the full capacity of the array to accommodate up to 384 unique ligands and the observation that the PGRN constructs tolerated multiple rounds of acid regeneration without detriment to their binding activity.

The Materials and Methods

Anti-hPGRN mAbs were sourced from the immunization of mice (in collaboration with Rinat-Pfizer) or chickens (in collaboration with Ligand Pharma, formerly Crystal Biosciences). The mouse mAbs were provided as conventional full-length IgGs with homodimer MW of 150 kDa, whereas the chicken mAbs were provided as a scFv fused to a human-IgG-Fc, where the scFv was from a wild-type or engineered chicken. The scFv-hIgGFc construct, therefore, had a homodimer MW of 100 kDa. All anti-PGRN mAbs were provided as purified material using standard protein A or G affinity chromatography. Mouse anti-His mAb (MAB050, R&D systems) was used as a sandwiching control. Recombinant purified human and mouse forms of PGRN, expressed with a C-terminal His-tag, were purchased from Sino Biologicals or R&D systems. A panel of five human/mouse chimeric PGRN constructs with His-tag were provided as crude material expressed in HEK293 Expifectamine conditioned media (Rinat-Pfizer).

Interaction Analysis

Surface Preparation

The LSA automates the choreography between two microfluidic modules for sample delivery onto a sensor surface; a single channel flow cell (SFC) and a 96-channel printhead (96PH). To prepare the surfaces for the binning experiment, the entire system (SFC and 96PH) was primed in a run buffer of 25 mM MES pH 5.5 + 0.01% Tween20. The SFC was docked onto a HC30M chip type and the entire chip surface was activated as a lawn by injecting a freshly mixed 1:1:1 (v/v/v) solution of 0.4 M EDC [1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce)] + 0.1 M SNHS (N-hydroxysulfosuccinimide, Pierce) + 0.1 M MES pH 5.5 (Carterra) in a final volume of 0.3 ml, for 10 min. The ligands (mAbs and antigens destined to be coupled) were diluted in 10 mM sodium acetate pH 4.5 and arrayed in a 96well plate; purified mAbs and antigens were prepared at 1-5 µg/ml while the crude chimeras (supplied in conditioned media) were diluted 15fold or 30fold. Samples were immobilized from a single 96 well plate in 4 immobilization steps to create a 384 ligand array. The 96-channel flow cell returns the samples to the sample plate after flowing, so the same samples were used to immobilize all 4 blocks. An 8-min contact time was used at each print location. After coupling, the SFC was docked over the printed array and excess activated esters were quenched by injecting 1 M ethanolamine HCl pH 8.5 for 8 min across the entire surface. The 96PH was returned to water (for cleaning), while the SFC, docked over the arrayed chip, was primed with a run buffer of HBSTE + BSA (10 mM Hepes pH7.4, 150 mM NaCl, 3 mM EDTA, 0.01% Tween20, 0.5 g/l BSA).

Analyte Preparation

To prepare the analytes for the binning experiment, the mAbs were diluted to approximately 20 µg/ml in HBSTE + BSA run buffer and arrayed in a 96well plate (0.28 ml/well). All analytes were dispensed into duplicate wells. 30 unique mAbs (the anti-PGRN mAbs and an anti-His mAb control) were used as analytes. The antigen (hPGRN) was prepared at 32 nM in a total volume of 20 ml (40 µg) for 80 binning cycles. 75 mM phosphoric acid was used for regeneration.

A classical binning script was used, comprising the following steps:

1. Baseline (1 min)
2. Capture Antigen (4 min)
3. Capture mAb Analyte (4 min)
4. Dissociation (1 min)
5. Regeneration (2x 30 sec)
6. Stabilization (1 min)

The run time was 20h based on an 80-cycle assay, using a 15-min binding cycle (above 6 steps plus needle wash routine after each injection). The data were analyzed in Carterra's Epitope software.

Data Analysis Using Carterra's Epitope Binning Software

Carterra has developed software that streamlines the analysis and presentation of several types of epitope characterization assays and represents a significant advancement in epitope competition data analysis. The software unifies complex steps into an integrated process including data quality control, signal normalization, cut-off (threshold) setting, and sorting of the competition matrix (heat map). While critical for data inspection and curation, viewing the competition matrix alone does not allow for the straightforward interpretation of complex data sets. For this reason, the epitope binning software takes visualization of these data to the next level with proprietary network and community plots, which allow the user to see and interact with the competition matrix.

Sensorgram data is always only a click away, regardless of the current processing step, allowing for a more detailed understanding of the potential nuances and complex behaviors such as "kick off" that only real-time label free binding data can provide.

Carterra's Epitope software enables epitope binning data to be processed quickly in a few steps and is compatible with premix or classical sandwich assay formats[1]. In the current example, a classical sandwich assay was employed **(Figure 1A)** in which a mAb analyte is injected over antigen that is first captured via the coupled mAb (the ligand array). **Figure 1B** shows an example of the sensorgram data viewed as an overlay plot for a single spot within the 384-ligand array, where the responses have been interspot referenced, Y-zeroed prior to the antigen injection for all cycles, and normalized to 1 at the end of the antigen capture step (the report point used for the normalization step is indicated by the green vertical bar). Normalizing the antigen-binding responses to 1 on a per cycle and per ligand basis facilitates a global analysis of the data. The binding responses used to determine sandwiching or blocking of the mAb analyte are indicated by the sandwiching report point, shown as an orange vertical bar in **Figure 1B**, which is set at the end of the mAb analyte binding step. This relative binding value compared to the response of a buffer blank mAb analyte (shown by the blue bolded sensorgrams) is used to populate the binning heat map. Global cut-offs are then set to determine the binding responses (Y-axis) of competing (blocked, in red) and sandwiching (not blocked, in green) analytes. Analytes falling between these two classifications are considered intermediate (ambiguous, in yellow).

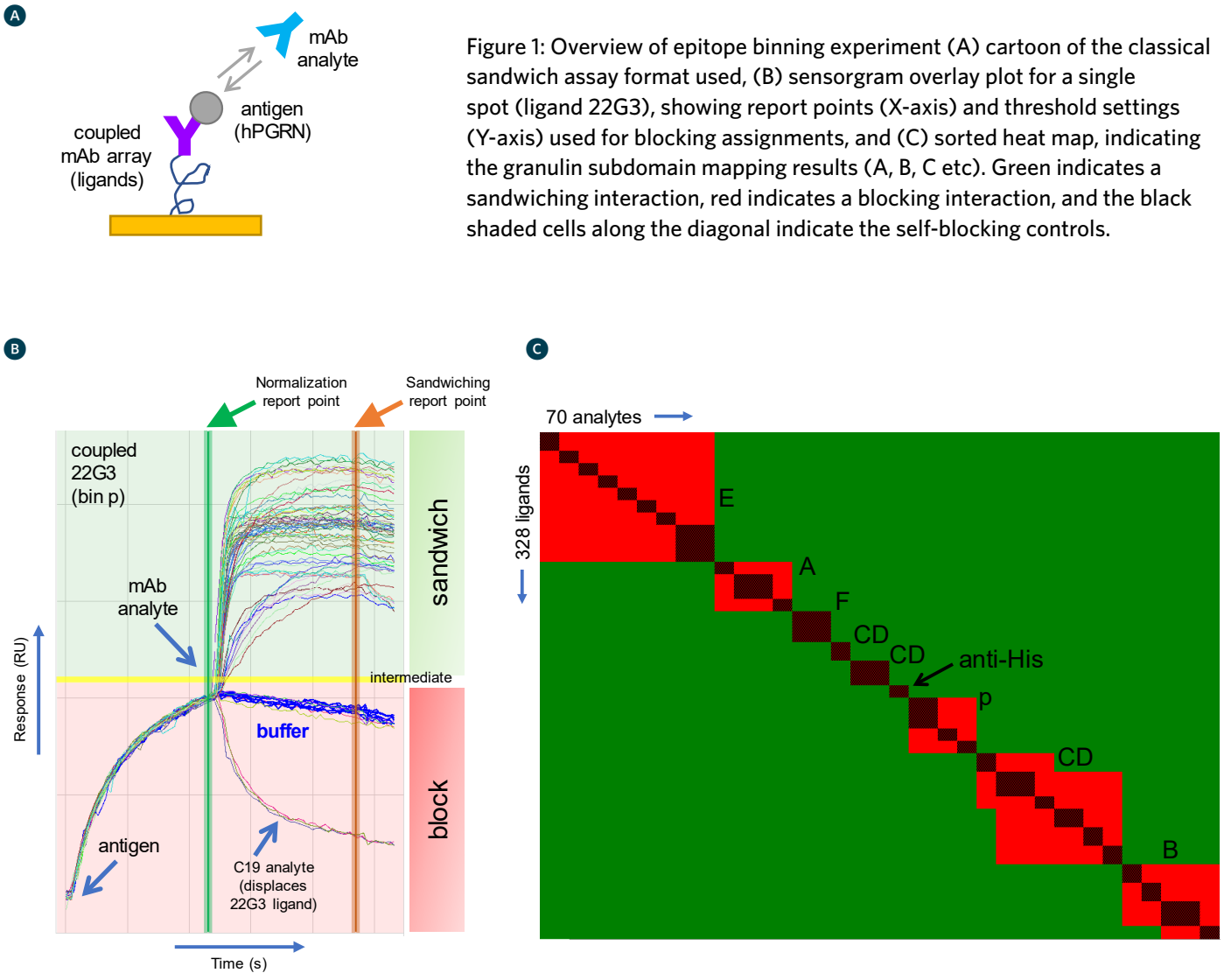


Figure 1: Overview of epitope binning experiment (A) cartoon of the classical sandwich assay format used, (B) sensorgram overlay plot for a single spot (ligand 22G3), showing report points (X-axis) and threshold settings (Y-axis) used for blocking assignments, and (C) sorted heat map, indicating the granulin subdomain mapping results (A, B, C etc). Green indicates a sandwiching interaction, red indicates a blocking interaction, and the black shaded cells along the diagonal indicate the self-blocking controls.

In the current example, a total of 22,960 discrete pairwise interactions were used to build the competition matrix shown in **Figure 1C**, comprising 328 ligands x 70 analyte cycles, excluding the interspersed buffer blank analyte cycles, used for threshold settings. This does not count the interspot references (unmodified regions of the chip in between the reaction spots) or the interactions from the coupled PGRN constructs (human, mouse, and chimeras) used to deduce the subdomain assignments. This experimental set up required the use of a total of 40 µg of hPGRN and approximately 5 µg per mAb (total use as both ligand and analyte). Note that the amount of each mAb required would not have changed regardless of the number of mAbs included in the epitope binning assay since each injection addresses the entire ligand array, which can accommodate up to 384 ligands. The coupling method took approximately 1.5h and the binning assay ran for approximately 20h unattended.

All mAbs in the PGRN set performed well as both ligand and analyte, and so all were included in the simplified bi-directional heat map in **Figure 2A**, where the results from each analyte/ligand interaction is distilled to a single cell. An alternate way of viewing the data is as a network plot, where the mAbs are shown as nodes and their blocking relationships are shown as chords. Envelopes inscribing node clusters define the bins. Networks are auto-generated and the Epitope Tool allows for the incorporation and visualization of orthogonal data as node color schemes **(Figure 2B)**.

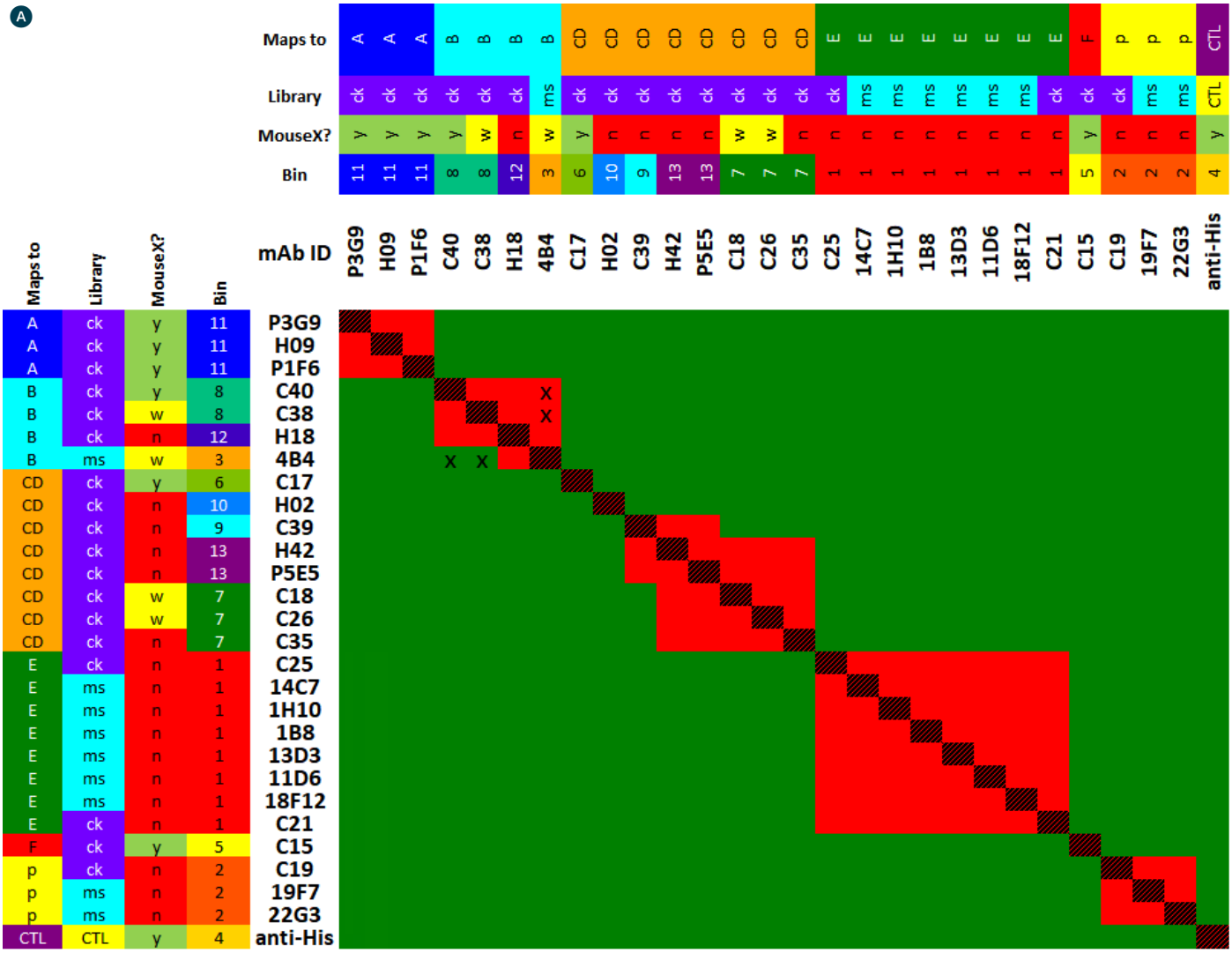


Figure 2A: Summary of binning results merged with orthogonal data: consolidated 28 x 28 heat map distilled to one cell per analyte/ligand interacting pair organized with orthogonal data (mapping, library, and mouse cross-reactivity). Cells marked X indicate an asymmetric binning result.

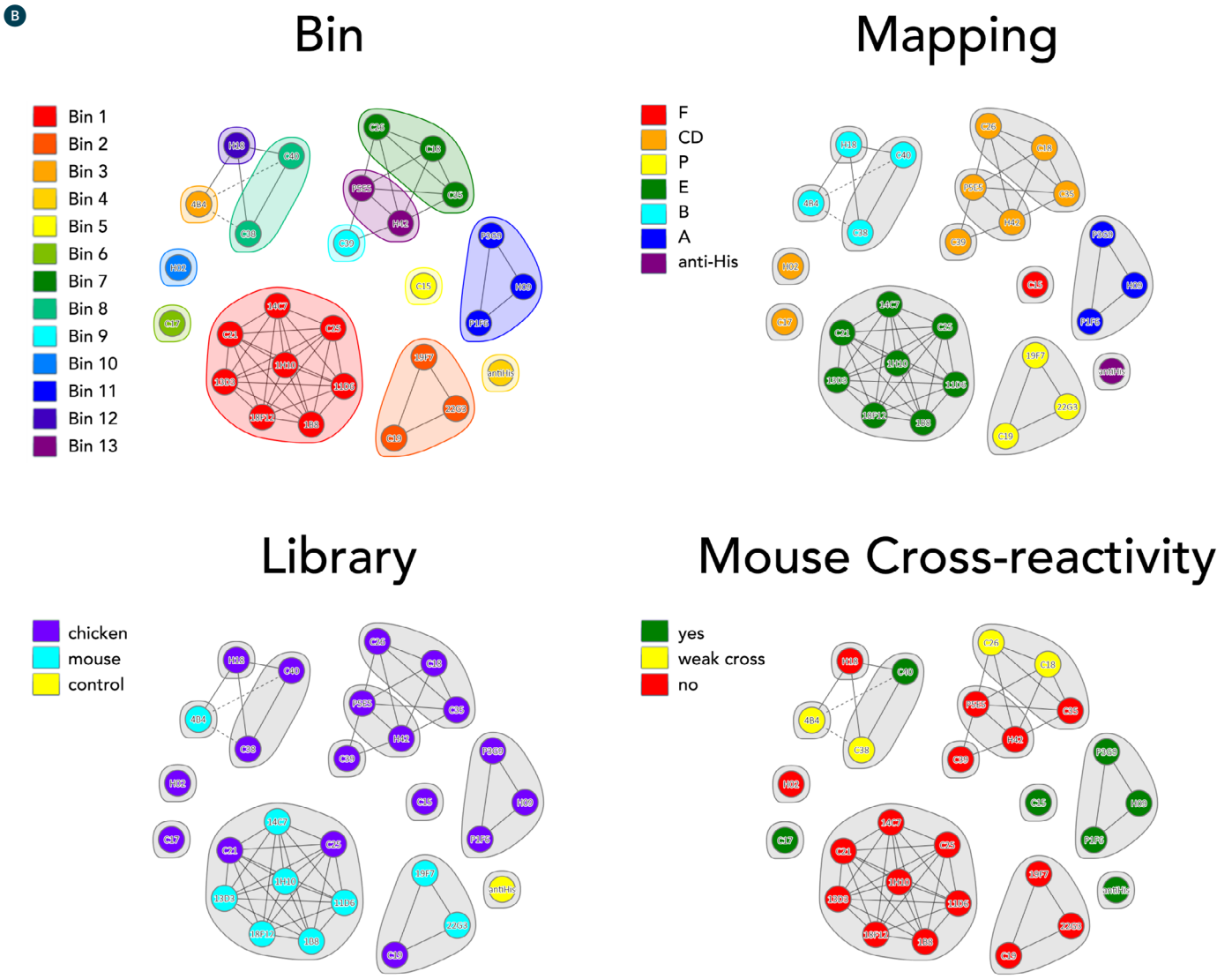


Figure 2B: Network plots colored by bin (default) or by orthogonal data.

Results

The networks show that the 30 mAbs fell into 13 unique bins **(Figure 2B)**. Several of the bins demonstrated shared but complex blocking relationships, such as the bin 7/9/13 cluster. Asymmetric competition was also seen between the bin 3 and bin 8 mAbs, shown by the dotted chord connecting these mAbs. This property of asymmetric competition is not believed to be an artifact of the assay format, but rather the classical sandwich assay format can reveal an allosteric type mechanism, where the binding of one mAb alters the availability of the epitope for another mAb, rather than binding to the exact same epitope[3]. An example is shown in **Figure 3A**.

Another interesting observation from this data set are clones that displace one another. An example is shown in **Figure 1B** by the inverted sandwiching response for C19 analyte when paired with coupled 22G3 (bin p). This effect is believed to occur when two antibodies target closely adjacent or minimally overlapping epitopes, resulting in the formation of a transient and unfavorable tri-molecular sandwiching complex (which may not be detected in the sensorgram) which rapidly dissociates to kick off or expel the ligand while leaving the antigen bound to the incoming mAb analyte[2]. In the C19/22G3 example, the formation of the tri-molecular complex is not obvious in the sensorgram data, but the precipitous dissociation effect is clear and manifests as an inverted sandwiching signal. This suggests that while they appear highly competitive in the binary

competition map, mAbs C19 and 22G3 bind closely adjacent, but non-identical epitopes. Another example of displacement was observed in bin E, where 14C7 (ligand) was displaced by all other bin E mAbs **(Figure 3B)**, suggesting that 14C7's epitope is closely adjacent to, but non-identical to the epitopes of other bin E clones.

The Epitope software also allows for alternate visual representations of the binning results **(Figure 4)**. The blocking relationships can be graphed as a dendrogram **(Figure 4A)**, where the tips represent the most granular analysis of the binning analysis, tolerating no blocking differences between the mAbs, resulting in the 13 bins reported in **Figure 2A**. Sliding the red bar up the Y-axis allows more differences in the blocking relationships to be tolerated, as shown by the chosen cut-height of 4.0, which results in 9 discrete epitope clusters, or communities, shown in **Figure 4B**. Thus, bins represent the most granular analysis of a binning experiment, whereas communities allow for a coarser analysis. Communities can be helpful, for example, if one is not interested in fine differences or complexities within a bin cluster and is more interested in reporting the number of discrete bin clusters.

Using no additional analyte injections, the binding of each mAb analyte to the directly coupled human, mouse, and five chimeric PGRNs was also measured **(Figure 5A)**. This example highlights the power and efficiency of the 384-ligand capacity of the LSA, since available spots on the chip can be coated with other proteins to provide additional information per analyte injection. Clones that recognized the human form of the receptor much more strongly than the mouse were mapped to subdomains via their pattern of recognition to the spotted chimeric PGRNs **(Figure 5B)**. Many of the anti-hPGRN mAbs did not cross react with the mouse ortholog and therefore could be mapped to specific granulins (**Figure 5C**, see C21 and H42). Mapping was also possible for the few clones that showed a weak cross-reactivity to the mouse ortholog. The mouse-crossreactivity of the anti-PGRN mAb panel is summarized in **Figure 2B**, as one of the color schemes. The mAbs that showed significant cross-reactivity to the mouse PGRN could not be directly mapped to a subdomain (**Figure 5C**, see C17), but their subdomains were inferred from their competition profiles, since mAbs showed blocking only within a subdomain and not across subdomains, consistent with the highly globular subdomain structure of PGRN. The multi-subdomain architecture of the protein is also implied in the binning results showing a series of discrete non-overlapping bins.

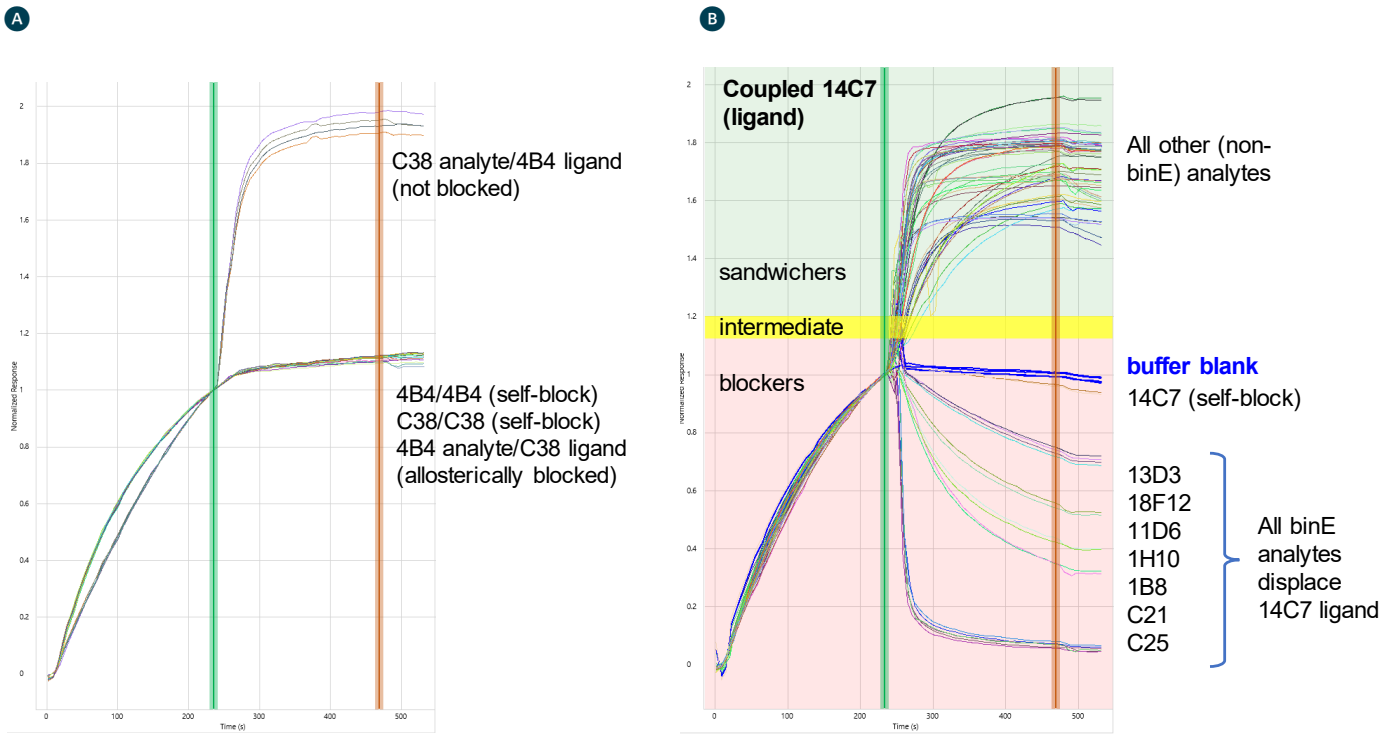
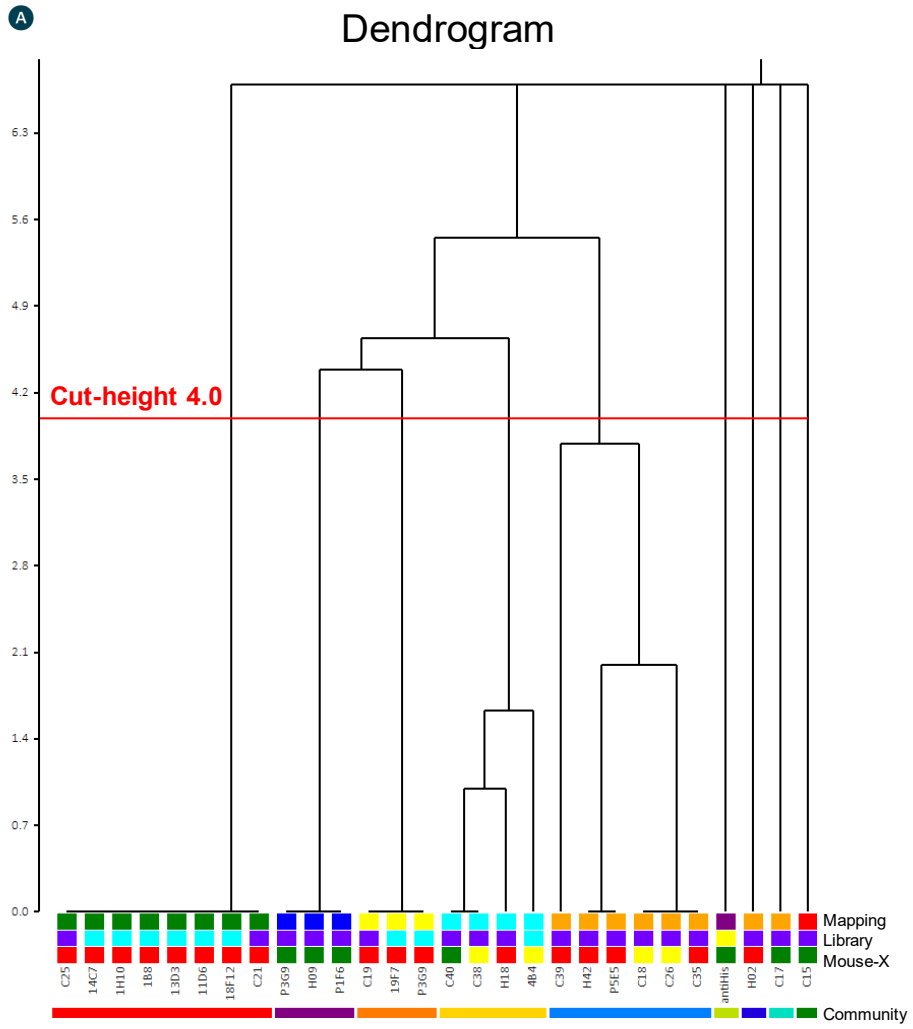


Figure 3: Examples of nuanced blocking profiles that can be easily identified using a classical sandwich assay format, (A) allosteric competition (asymmetric block) and (B) displacement.



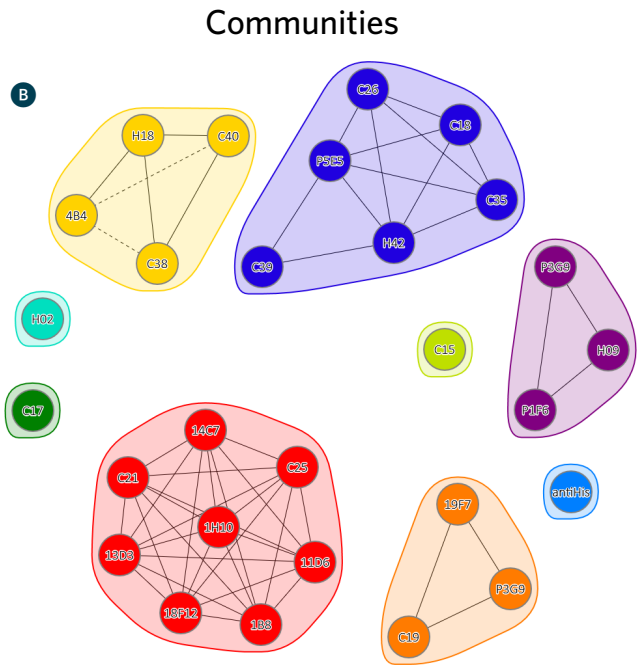


Figure 4: Alternate representation of the binning results, (A) binning dendrogram, where the tips show zero blocking differences tolerated between mAbs, giving the 13 bin assignments shown in Figure 2B. By adjusting the cut-height, a coarser clustering assignment is generated, giving communities. (B) Community plot, showing 9 distinct clusters, consistent with the dendrogram's user-defined cut-height.

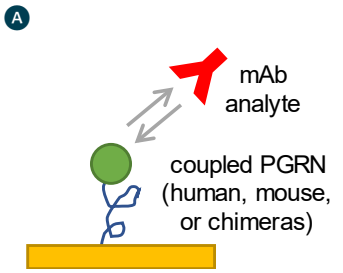
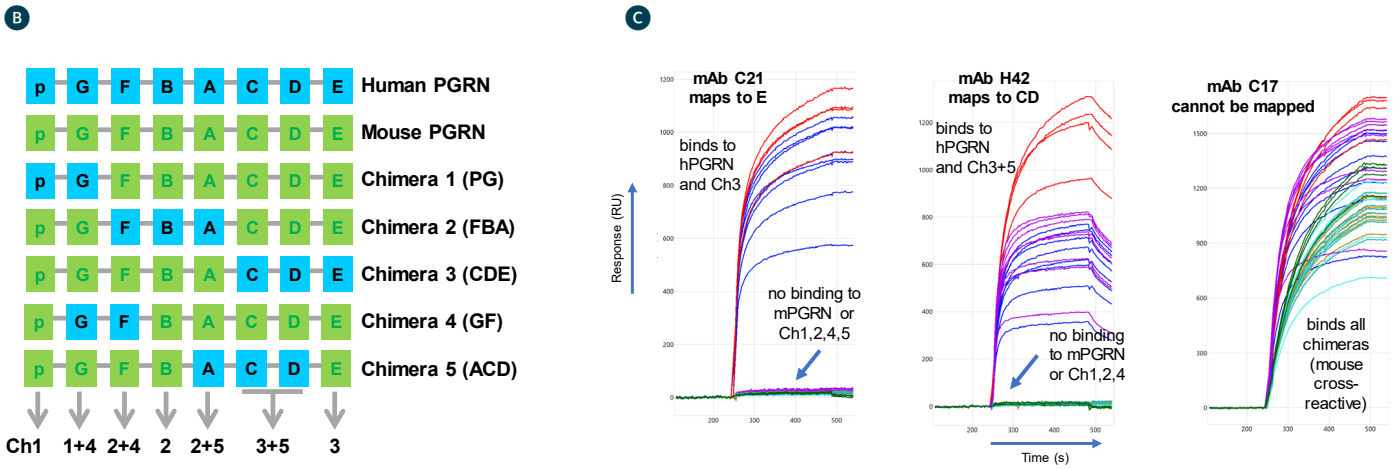


Figure 5: Epitope mapping using a chimeric swap strategy (A) Composition of the human/mouse chimeric PGRN constructs used to deduce the specific subdomain or granulin targeted by each mAb, (B) Cartoon of the assay format used, and (C) sensorgram overlay plots (grouped by mAb analyte) for mAbs that target different granulins, shown in 4 to 8 ligands spots each. The binding specificity to a subdomain was deduced from the construct design shown in panel A. For example, mAb C21 bound only the human PGRN (red) and chimera 3 (blue), so was mapped to granulin E, whereas mAb H42 bound chimeras 3+5 (blue and purple respectively), so was mapped to granulin CD. Since mAb C17 was mouse cross-reactive (green), it bound to all chimeras and was not amenable to mapping by this strategy.



Summary

The PGRN mAbs described here provide a useful model set of reagents to demonstrate and train users on how to run and analyze high-throughput SPR epitope binning systems. Carterra's LSA serves as a highly efficient and facile biophysical tool for performing high throughput epitope binning. This 28 x 28 matrix, performed as a 384-ligand array to provide replicate spot-to-spot measurements, and as a duplicate analyte series to provide repeat injections, to overall enhance the robustness and statistical confidence in the binning results, was set up with minimal hands on time and a simple set-up which only required two 96 well plates, one with the immobilization mAbs and PGRN constructs and another with the analyte samples. Also, given the highly parallel nature of the array, an orthogonal chimeric sub-domain mapping strategy was achieved with no additional assay steps or assay cycles.

References

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