# Chapter 19

# Competitive Epitope Binning Using HT-SPR

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## Abstract

Competitive epitope binning using high-throughput surface plasmon resonance (HT-SPR) is a method for understanding competitive epitope profiles of up to hundreds of monoclonal antibodies. Detailed here is an approach along with considerations for the design and analysis of a classical competitive epitope binning assay using the Carterra LSA. Monoclonal antibodies are arrayed on a biosensor chip and interrogated serially for their ability to form a trimolecular complex with antigen and solution phase monoclonal antibodies. Software tools allow the clustering of monoclonal antibodies sharing common epitopes, and the real-time nature of the data allows for study of binding profiles for each monoclonal antibody.

**Key words** HT-SPR, Biosensors, Monoclonal antibodies, mAbs, Competitive epitope binning, Classical sandwich binning, Premix binning

## 1 Introduction

Since the launch of the first mAb-based therapy in 1986 [1], the market size of mAb or mAb-like formats has grown rapidly and is expected to reach nearly $500 billion by 2028 [2]. Every top 20 pharmaceutical company and hundreds of biotech companies across the globe leverage mAbs as part of their therapeutic strategy. From a strategic standpoint, epitope binning is highly valuable for a number of reasons:

- Functionality and MOA of a therapeutic antibody are linked to its epitope.
- Epitope is innate to a mAb, cannot be readily engineered, and must be screened or selected.
- Epitope binning is deployable as an early-stage surrogate for functional diversity.
- Through binning large sequence sets can be related to functional classes.
- Epitope binning enables the establishment of IP and differentiation from other molecules.

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With limited tools and throughput, historically epitope binning was a strategy reserved for small numbers of mAb candidates in the later stages of drug discovery [3]. This was problematic because characterization then occurred at a point in the process when candidate diversity was likely low, and barriers existed to returning to select again from broader candidate pools. The bottleneck began to change with technologies focused on increasing the numbers of mAbs that could be epitope binned; *see* **Note 1**. As biosensor throughput has scaled in conjunction with antibody discovery technologies, epitope binning has emerged as a frontline tool in modern and sophisticated biotherapeutic strategies [4–6]. Even more recently epitope binning is expanding beyond informed selection of viable drug candidates to also being leveraged in AI−/ML-dedicated workflows where it helps in both the development and validation of predictive models [7–9].

Competition-based epitope binning is an assay format where pairs of monoclonal antibodies (mAbs) are tested for their ability to form a ternary complex with their respective antigen (Ag). mAb samples are injected in a full factorial assay design; e.g., for two mAbs, A and B, the following pairwise injections are performed: A-A, A-B, B-A, and B-B. If two mAbs can bind the same Ag simultaneously, it suggests they do not share overlapping epitopes and are sandwiching. Conversely, if both mAbs cannot simultaneously bind to the same Ag, this suggests they both recognize a similar, if not identical, epitope and are considered to be competitive. This process can be conducted for as few as a single pair of mAbs and as many as hundreds of mAb pairs per experiment. This process is not necessarily restricted to intact mAbs, and additional biotherapeutic formats such as peptides, aptamers, dAbs, scFvs, and nanobodies can be assessed in the same manner. Additional competitors such as natural receptors and ligands or other interacting proteins can be included in the analysis. Figures 1, 2 and 3 show the common competitive binning formats; *see* **Note 2** for more details.

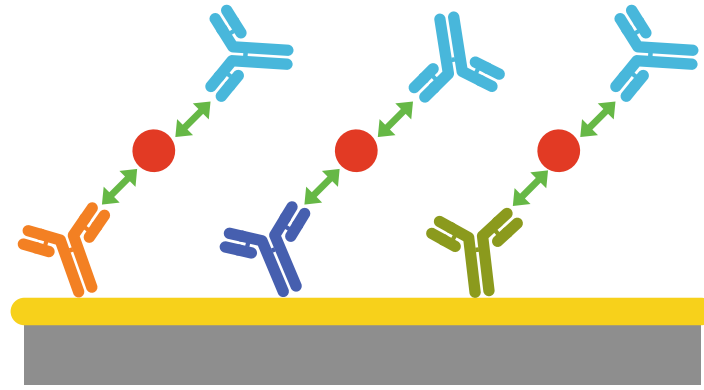

**Fig. 1** Classical sandwiching assay format

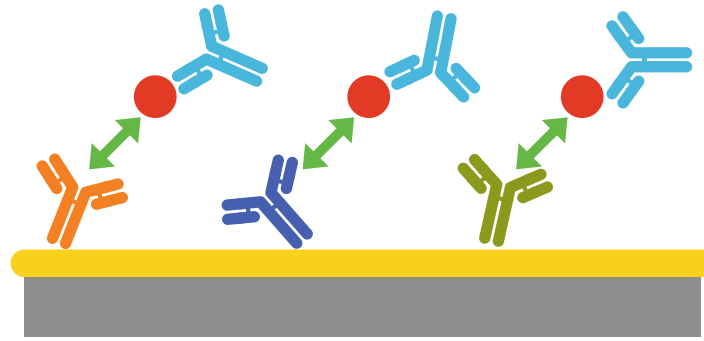

**Fig. 2** Premix binning format

**Fig. 3** In-tandem binning format

It is important to consider the underlying molecular complexity when conducting and evaluating competitive epitope binning experiments; *see* **Note 3**. Furthermore, consideration must be given to how competitive epitope binning fits into a workflow, including sample numbers and availability as well as overall assay efficiency; *see* **Note 4**. It's worth noting that competitive epitope binning should be distinguished from another, albeit less common, approach to epitope characterization: epitope mapping. A brief description of epitope mapping can be found in **Note 5**. This chapter will discuss in detail the methods and analytical approach of classical competitive epitope binning using high-throughput SPR (HT-SPR) for experiment execution and Epitope software for data analysis of a panel of purified mAbs.

## 2 Materials

- Carterra LSA or LSA$^{XT}$ Instrument.
- Carterra Epitope analysis software.
- Sensor chip (HC30M).
- 25 mM MES + 150 mM NaCl + 0.05% Tween, pH 5.5.
- 10 mM sodium acetate pH 4.0, 4.5, and 5.0.

- 1× HBSTE buffer (10 mM HEPES + 0.05% Tween + 150 mM NaCl + 3 mM EDTA, pH 7.4).
- Bovine serum albumin (BSA; use IgG-free form).
- 10 mM glycine pH 2.0.
- 100 mM MES pH 5.5.
- 10% Tween-20.
- 400 mM EDC.
- 100 mM sulfo-NHS.
- 1 M ethanolamine.
- Soluble monovalent antigen (Ag).
- mAbs raised against the Ag.
- 96-deep-well plates.
- 1.5 mL vials.
- 50 mL conical tubes.

## 3 Methods

Key to executing a robust competitive epitope binning assay is consistent Ag binding from cycle to cycle. Immobilization and regeneration conditions should be optimized to ensure the surface-bound mAbs (ligands) remain stable over the course of the experiment to avoid false positives or negatives. In HT-SPR binning assays, there can be hundreds of cycles executed to develop a complete competition profile for all clones. Optimizing the assay ensures that over the course of the experiments, the reagents perform as expected and that if there are issues, they can be easily identified. Figure 4 lists the major steps in optimizing and executing a competitive epitope binning assay using the classical format as an example.

### *3.1 Immobilization via Amine Coupling*

1. Remove HC30M sensor chip from −20 °C storage and allow to warm for at least 15 min.
2. Dock the HC30M sensor chip in the Carterra LSA or LSA$^{XT}$.
3. Set the interaction thermals to 25 °C and the sample deck temperature to 15 °C. Prime the fluidics in 25 mM MES pH 5.5 + 150 mM NaCl + 0.05% Tween-20.


**Fig. 4** Steps in executing an HT-SPR epitope binning experiment

4. Prepare samples in 10 mM sodium acetate coupling buffer, at pH 4.0, 4.5, and 5.0. mAb concentrations from 1 to 5 μg/mL are recommended. The multichannel fluidics of the Carterra LSA and LSA$^{XT}$ allow for up to 96 conditions to be tested in parallel. Running a multichannel quant experiment in the Navigator control software allows for viewing of preconcentration responses to the chip surface. *See* **Note 6** for more details.
5. Following preconcentration scouting, the mAbs are prepared in the optimal coupling buffer and concentration conditions, 250 μL final volume of each in a 96-deep-well plate. Concentrations as high a 15 ug/mL can be used to ensure robust antibody loading.
6. For the quenching solution, prepare 300 μL of ethanolamine in a 1.5 mL vial.
7. Prepare wash solution 300 μL of 1× HBSTE in a 1.5 mL vial.
8. Prepare activation solution by combining 200 μL of 100 mM MES pH 5.5 and 100 μL each of 400 mM EDC, and 100 mM sulfo-NHS in a 1.5 mL vial. Final concentration is 100 mM EDC, 25 mM S-NHS.
9. Load plate of mAbs along with vials into the instrument and shut the doors.
10. Select the Surface Prep Array method and enter 7 min for the activation injection, 10 min for the mAb coupling, 8 min for the ethanolamine quenching, and 2 min for the HBSTE wash.
11. After running the experiment, observe the starting baseline of the experiment relative to the bound levels of mAb to determine the amounts in response units (RU) coupled to the surface.
12. If working with non-purified mAbs, *see* **Note 7**.

### 3.2 Antigen Concentration and Regeneration Scouting

1. *See* Table 1 for some common regeneration solutions.
2. Prime the instrument in 1× HBST or 1X HBSTE + 0.5 mg/mL BSA.

**Table 1**
**Typical regeneration solutions for competitive epitope binning using HT-SPR**

| Solution | Typical pH |
|---|---|
| 10 mM glycine HCl | 1.5–2.5 |
| 0.02–0.05 M sodium hydroxide | 10–11 |
| 0.85% phosphoric acid | 1.7 |

Up to 1 M NaCl and/or 1% Tween-20 can be added to glycine when pH alone is insufficient

3. On a deep-well plate, prepare a titration series of Ag from 300 nM in threefold serial dilution with five concentrations (300 nM, 100 nM, 33.3 nM, 11 nM, 3.7 nM), with a minimum final volume of 300 μL/well. Also fill nine wells with 300 μL running buffer.
4. Prepare 1.5 mL Ag at 50 nM in HBSTE-BSA buffer in a 15 mL tube.
5. Add 1 mL of regeneration solution to a 1.5 mL vial. Start regeneration testing with a mild condition; 10 mM glycine pH 2.0 is a good choice for initial binning evaluation.
6. Create two methods. The first will be injection cycles with no regeneration, 5-min association and 10-min dissociation. Inject six to 8 cycles of buffer from wells followed by the Ag concentration series from low to high. This run will allow for the kinetic evaluation of all of the immobilized clones and to understand the required Ag concentration for use in the binning. The second method is a series of injections with regeneration, three cycles of buffer injections followed by four injections of 50 nM Ag for 5 min, followed by regeneration for 30 s with one of the glycine solutions (pH 2.2 or 2.0 are good first choices).
7. Queue the methods and run. The injection of the concentration series of Ag, performed before any regeneration, informs the starting activity of all the immobilized ligands and allows for one to determine the concentration required for use in the binning assay (*see* **Note 8**). Depending on surface densities of ligands, estimates for the kinetic rate constants from this binding may also be determined.
8. Next compare the bound Ag levels in each cycle of the regeneration experiment and the baseline. If the Ag binding capacity of the ligand mAbs is consistent for the last three of the four cycles and the baseline is stable, the regeneration condition is suitable for the binning. If the regeneration is incomplete, longer contact time or more acidic conditions are likely required, and the regeneration test can be repeated using different conditions until suitable ones are found.
9. Additionally, confirm Ag responses are >50 RU. If below, adjust Ag concentration used in the final assay. *See* **Note 8**.

### *3.3 Classical Epitope Binning Experiment Execution*

1. In a shallow 96-well plate, prepare the mAbs at 30 ug/mL (200 nM) using HBSTE-BSA running buffer with a final volume of 290 μL. *See* **Note 9**.
2. Prepare Ag to the concentration determined during scouting in the running buffer. For each cycle 270 μL will be injected; therefore, view the injection table to count the number of

cycles needed and multiply by 270 μL. Be sure to include warm-up cycles (typically 3) and periodic control injections (every 12 cycles), in a 50 mL conical tube (e.g., 29 mL for a 96-analyte binning which totals 105 cycles).

3. Add an equivalent volume of the regeneration solution determined during the scouting exercise to a 50 mL tube (>270 μL × the cycle number).
4. In the instrument control software, select the Classical Binning method and populate the tables for the ligand and analyte names. Set the Ag injection time for 5 min and the mAb injection time for 5 min. Specify the regeneration injection time determined during the scouting exercise.
5. Ensure there is sufficient assay buffer and that the waste carboy is empty; then select Run.

### 3.4 Basic Data Processing

1. *See* **Note 10** for additional details.
2. Once the run has completed, open the .sprdata file using the Epitope software.
3. On the Analyte Processing page under Reference, inspect the reference ROI signals for evidence of nonspecific binding, which often manifests as responses that fail to return to baseline immediately after the injection completes. If no evidence of nonspecific binding is present, subtract the reference signals. *See* Fig. 5.
4. The regeneration pulse signals are unnecessary for analysis. To remove select the range of data to exclude under the Crop tab and apply. *See* Fig. 6.
5. On the Y-Align tab in the Serial View, position the y-align bars in the center of the baseline for the first cycle and select Y-Align. Observe the baseline for each cycle; some slight drift is normal, but if the baseline incrementally increases in each cycle, it suggests insufficient regeneration. If the baseline is stable, but the Ag injections decrease in magnitude, it suggests loss of ligand activity. Slight inactivation of ligands over the course of the experiment can be compensated for in subsequent steps, but if there is insufficient regeneration, consideration should be given to re-running the experiment under more optimal conditions. If regeneration looks stable, y-align all cycles at the baseline in the Overlay view. *See* Fig. 7.

### 3.5 Classical Epitope Binning Data Analysis

1. For the next two steps, refer to Fig. 8 and **Note 11**.
2. Following the standard data processing steps in the Epitope software, on the Binning tab, position the Normalization bar near the end of the Ag injection. This scales the data uniformly

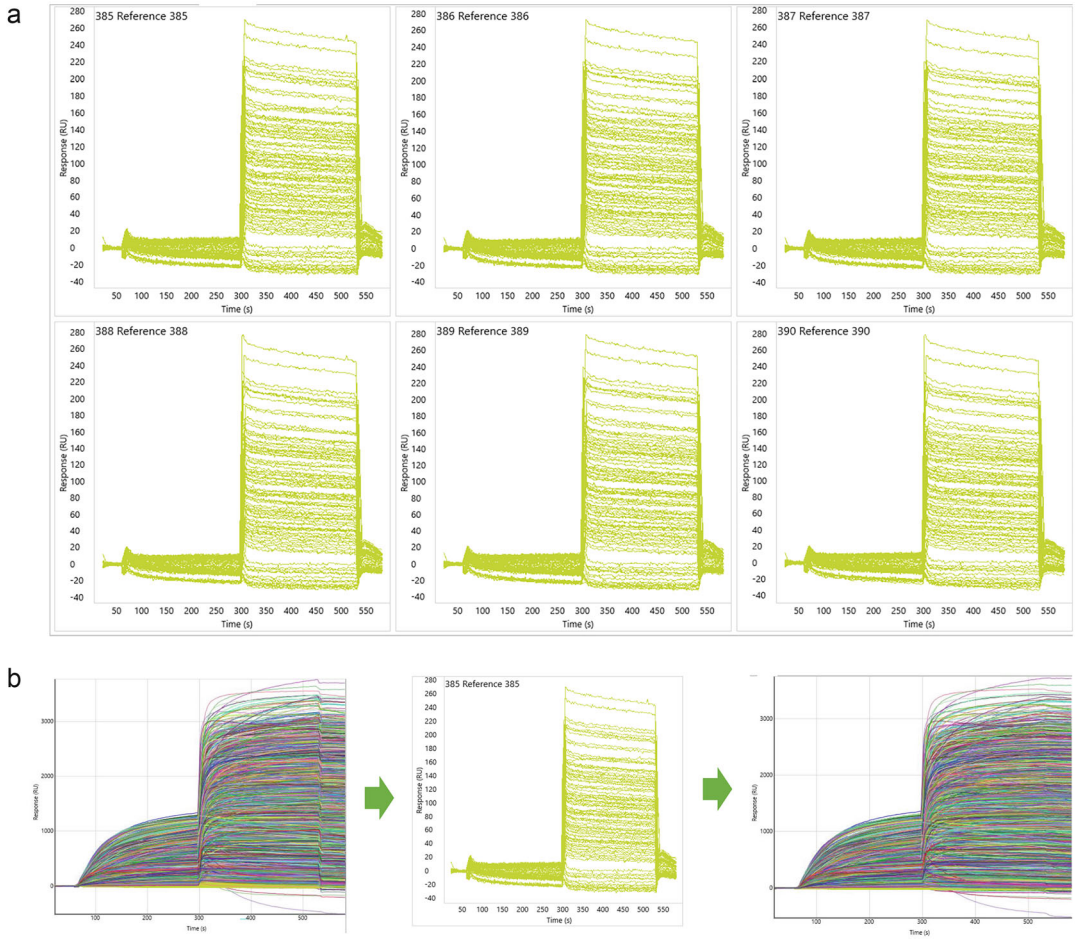


**Fig. 5** Referencing. (**a**) Reference profiles for six reference surfaces. (**b**) Data before and after reference signals are subtracted

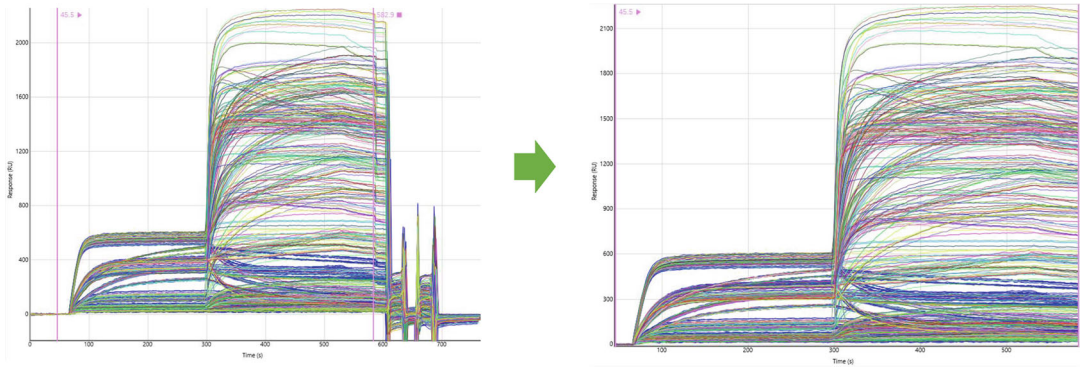

**Fig. 6** Cropping of sensorgrams

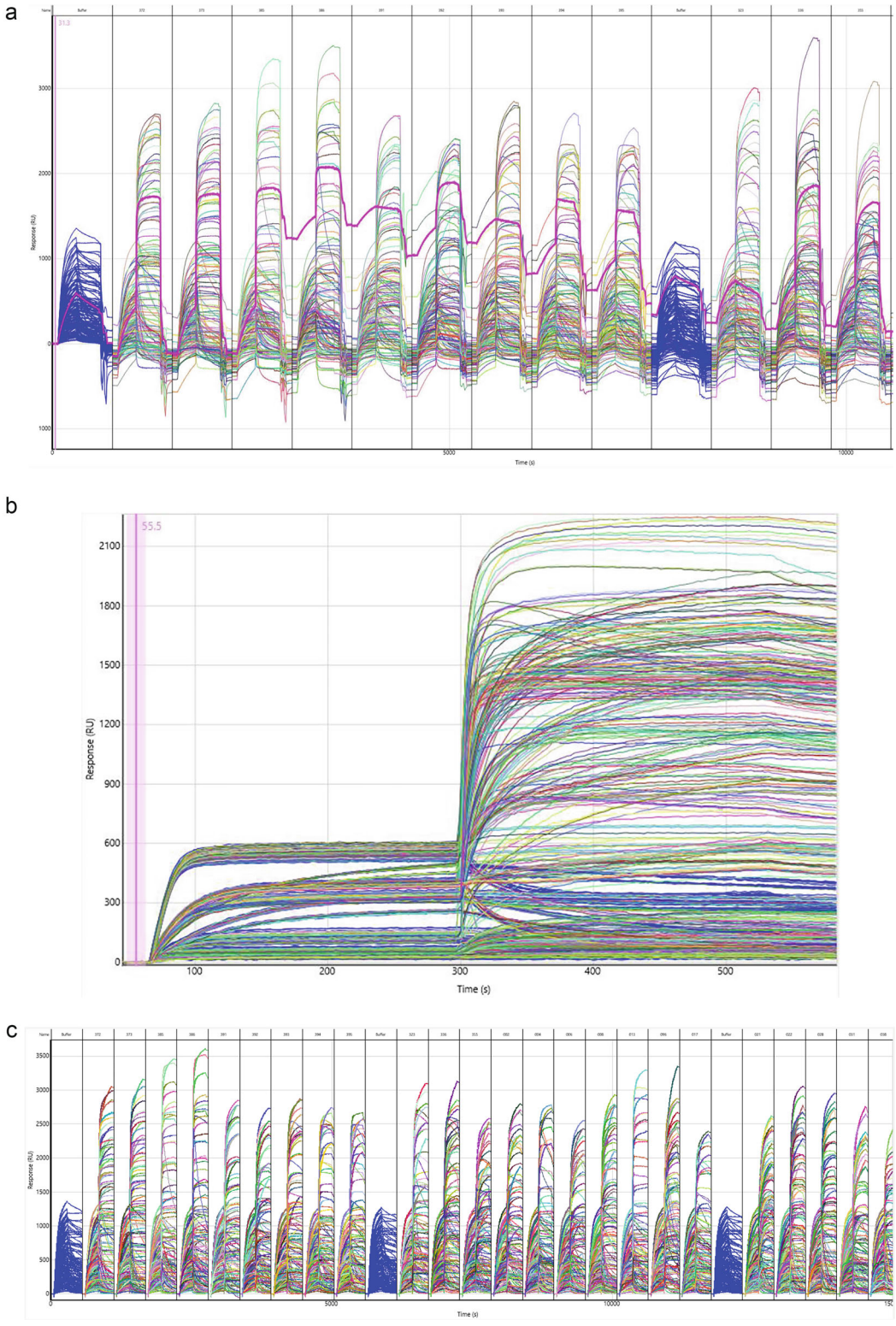


**Fig. 7** Y-alignment. (**a**) Binding responses during an experiment using serial y-alignment. Note the sensorgram highlighted in pink showing an example of incomplete regeneration. (**b**) Location and application of y-alignment during the baseline of each cycle, in overlay view. (**c**) Serial view of sensorgrams following global y-alignment

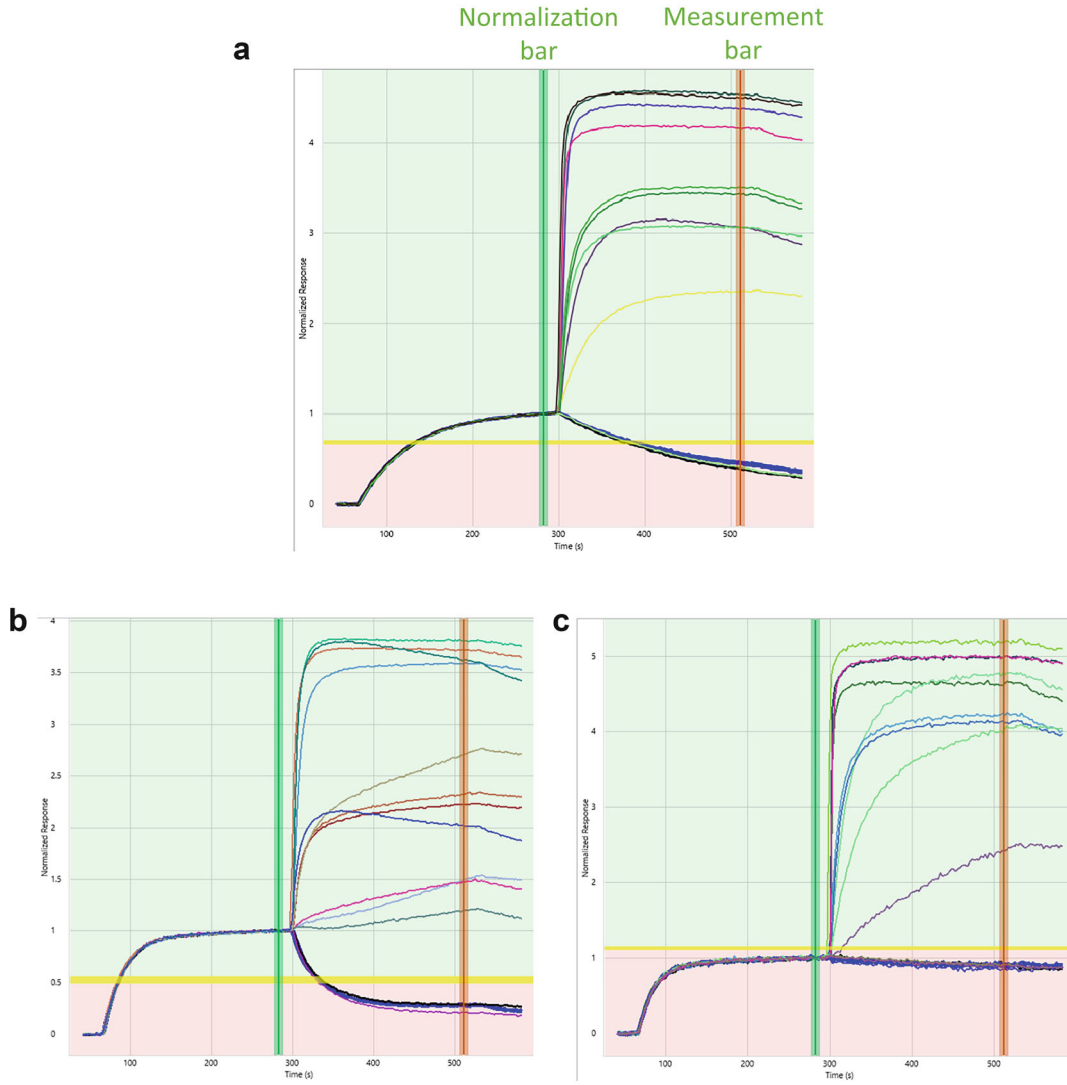


**Fig. 8** (**a**) Vertical green normalization bar and orange measurement bar positioned on sensorgrams for a single mAb ligand. (**b**) Rapid dissociating surface. (**c**) Slow dissociating surface

and accounts for any changes relating to decreased Ag binding over the course of the experiment.

3. Position the Measurement bar near the end of the mAb injection. This location is then used to calculate the magnitude of mAb sandwiching relative to the Ag-only control injections.
4. Under the QuickClean menu are options for removing ligands which fail to bind sufficient Ag as well as those that fail to self-block. Highlighting and removing these ligands will significantly improve interpretation of the competitive outcomes. *See* Fig. 9 and **Note 12** for more details.

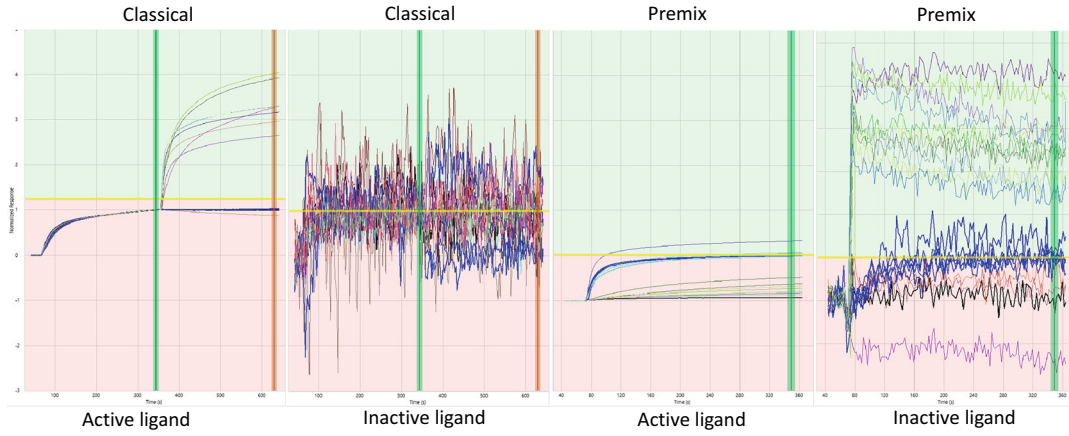


**Fig. 9** Ligand curation. Note: Inactive ligands show high noise in normalized data, as the sensorgrams with no binding are being normalized to the same scale as a real binder. You can view non-normalized data as well using the options

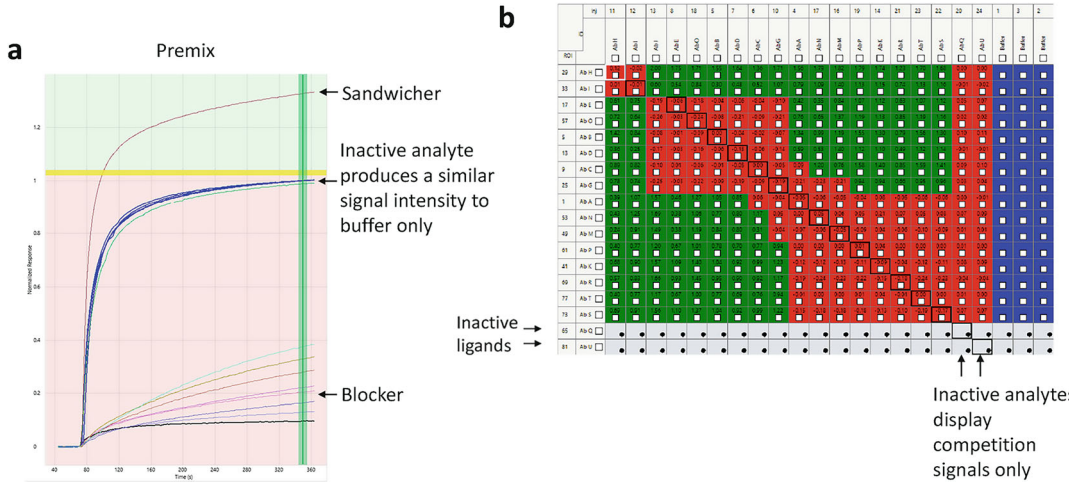


**Fig. 10** Analyte curation

5. Analyte injections that exhibit broad sandwiching, especially with themselves (Fig. 10a), or those that are largely blocking (Fig. 10b), should be carefully investigated and possibly removed. *See* **Note 13**.
6. The competition threshold bar (horizontal yellow bar Fig. 11) delineates sandwichers from blockers. In addition, by adjusting its thickness, it can also classify interactions that are considered intermediate. The bar can be adjusted for all ligands globally or else on a per ligand basis. *See* Fig. 11 and **Note 14**.
7. After reviewing the competition threshold settings for the ligands, the heat map should be sorted and studied for patterns

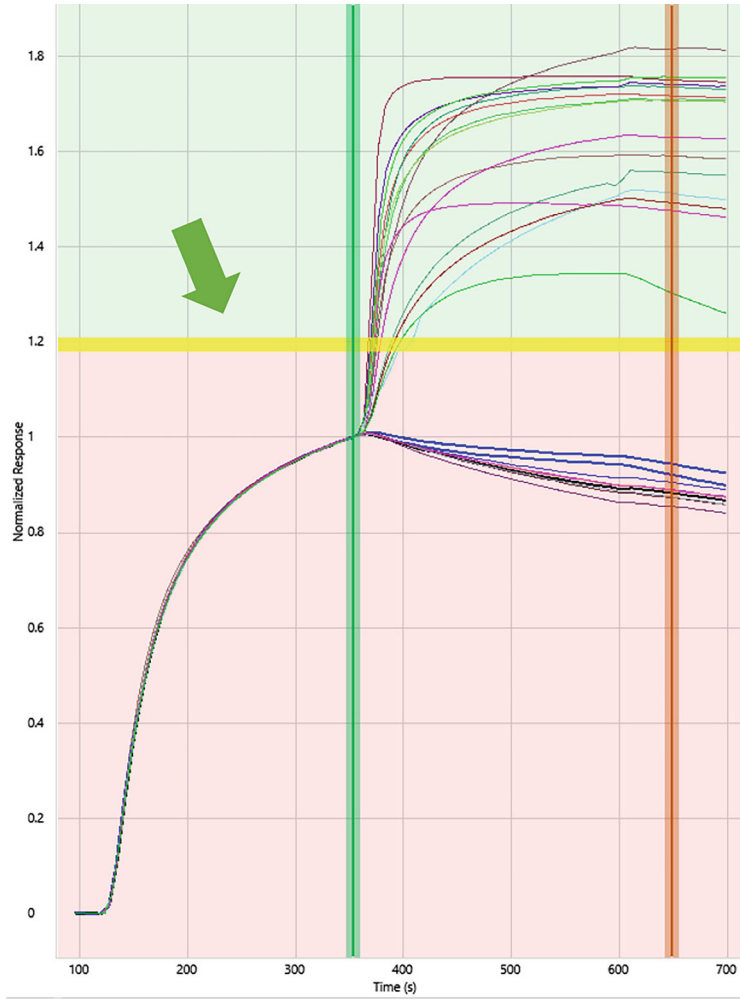


**Fig. 11** Competition threshold is set with the yellow line. Note the value recorded is the different between the controls and actives directly under the orange bar

suggesting additional curation is needed or if any unusual behaviors are present. Selecting the "Show Asymmetry" option allows highlighting of interactions where sandwiching or blocking is not equivalent for both orientations of a mAb pair (Fig. 12). By studying heat map symmetry and then investigating sensorgram profiles, more complex behaviors such as allostery or kinetic modulation can be observed, though confirmation of these behaviors likely requires additional experiments (Fig. 13). *See* **Note 15**.

8. After thresholds have been confirmed and symmetry investigated, the data can be sorted to create the dendrogram and associate like clusters. The heat map will be sorted to match the dendrogram order (Fig. 14 and **Note 16**).

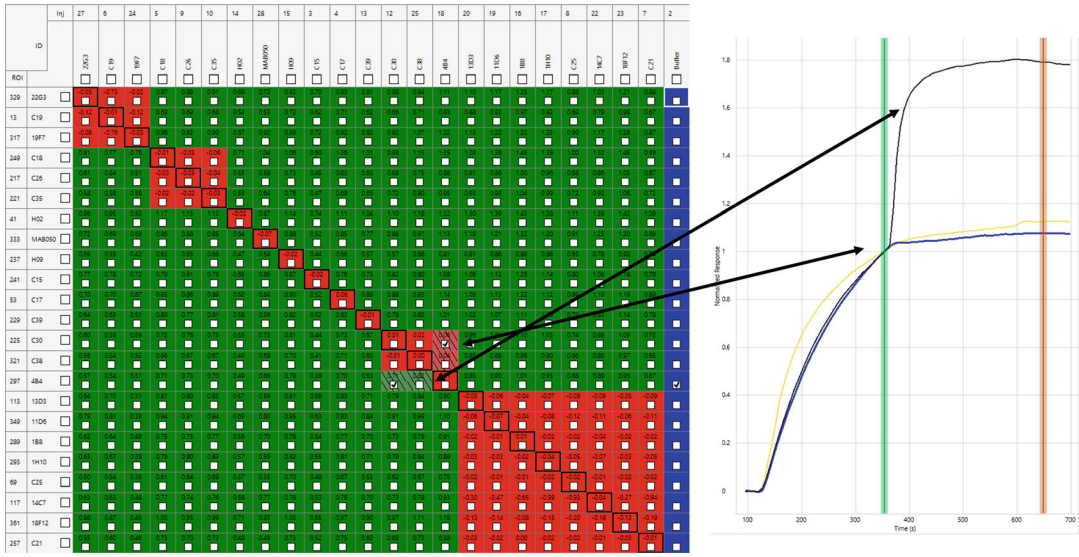

**Fig. 12** Asymmetry. In this example two asymmetrical interactions are highlighted. The sandwiching pairs show opposite classification in ligand and analyte orientation (hashed cells). The related sensorgrams can be displayed, showing the strong sandwich in one orientation and very limited response in the other

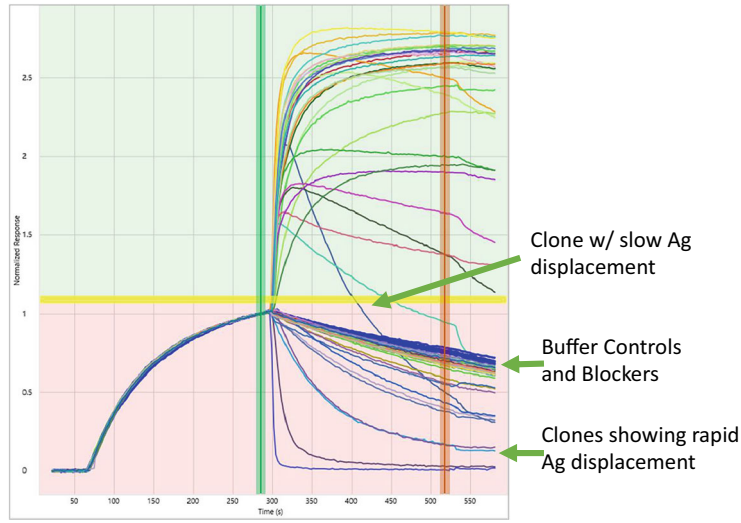


**Fig. 13** Allostery and kinetic modulation. Sensorgrams with rapid displacement and slow displacement are shown

9. Network plots relating to this subsequent sandwiching/blocking assignments and clustering are generated by selecting the Network icon.
10. The last major step in curating the competitive binning data set is to adjust the variable cut height bar (horizontal red bar Fig. 15) of the dendrogram which dictates how granular the clustering will be on the community network plot. Cut heights

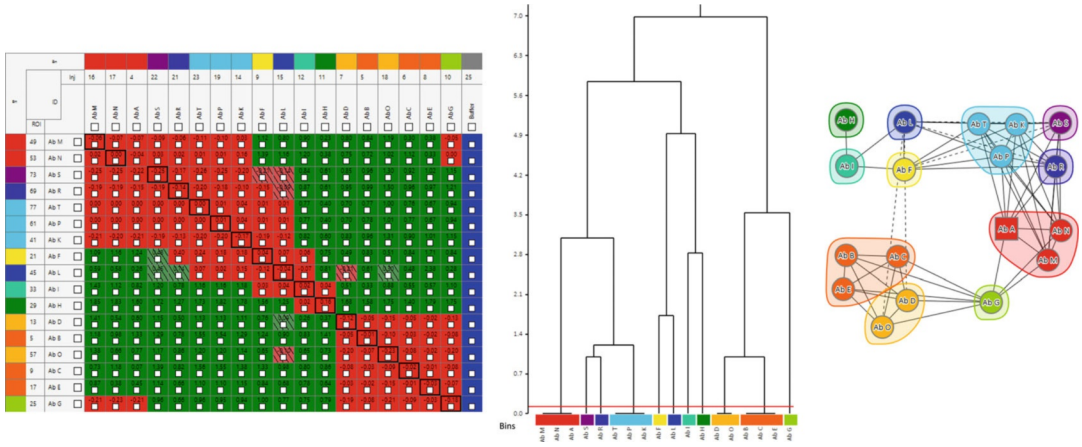

**Fig. 14** Heat map, Dendrogram, and Network plots. In the heat map rows are the immobilized mAbs and columns are the injected analyte mAbs. Cells are red and green, indicating sandwiching and blocking respectively, and self vs. self is bold outlined. The dendrogram in the center shows a hierarchical clustering tree with branches representing the shared blocking relationships between the mAbs. The network diagram on the right illustrates interconnected nodes, grouped by color as epitope bins, indicating the identical sandwiching and blocking profiles

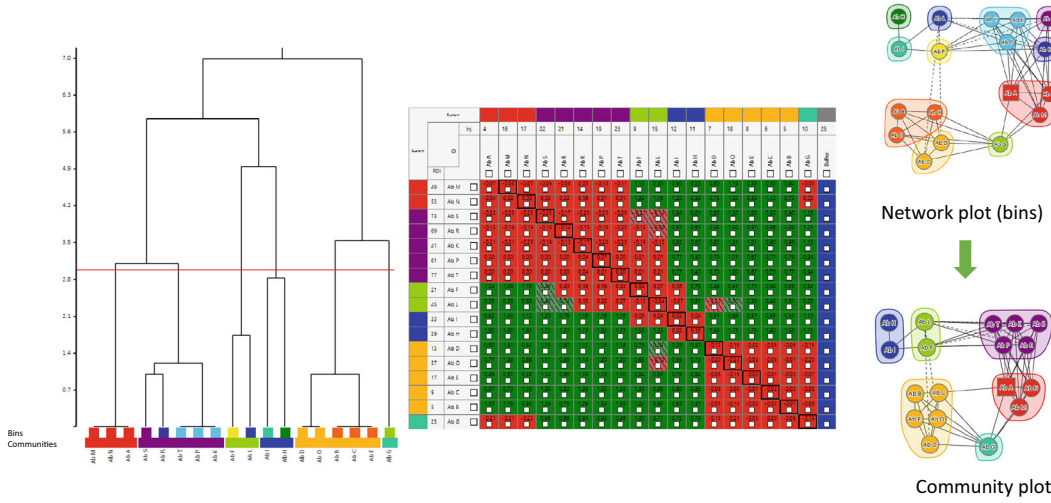


**Fig. 15** Dendrogram and Communities. A dendrogram on the left shows hierarchical clustering of bins with color-coded communities at the bottom. The position of the red community threshold lines shows the height in the dendrogram where the communities are assigned. The heatmap displays the full matrix of interactions and is sorted in the same order as the dendrogram. Columns and rows have a border cells colored to reflect the assigned community. A network plot is transitioned into a community plot, grouping related bins into communities

should be chosen that group similarly competitive clones while avoiding grouping of clones with little to no shared relationships. Further details on choosing appropriate cut heights can be found in Figs. 15 and 16 as well as **Note 17**.

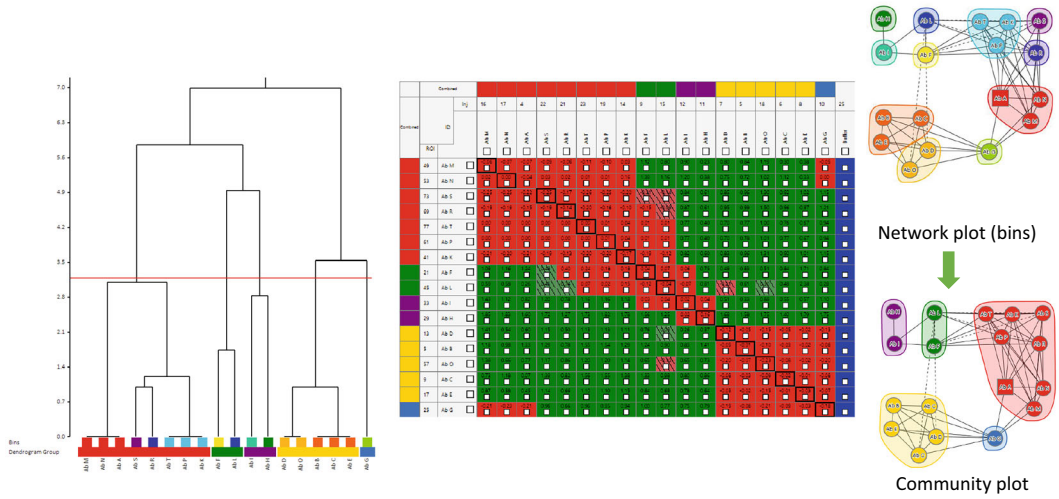


**Fig. 16** Dendrogram and communities with higher clustering threshold

## 4 Notes

1. *Surface plasmon resonance (SPR):* SPR-based biosensors were first commercialized in the early 1990s by Pharmacia Biotech, under the product name BIAcore. SPR instruments rely on detection of changes in light absorption and reflectivity corresponding to mass effects (refractive index) on an evanescent wave in the thin metallic layer (surface plasmon) at the sensing surface. SPR systems are sensitive to changes in the refractive index of the flowed solution, so proper signal referencing and considerations of buffer and sample matching are required and well understood. The first broadly commercially available systems offered four sensing surfaces, termed flow cells. Since then, multiple different configurations of flow cells have been made available from 2 flow cells up to 8, with various configurations allowing for up to 36 interactions to be monitored in parallel. These SPR devices span the range from very simple devices that are operated manually to systems with fully integrated liquid handling that can run unattended for a week or more. Common to all commercial SPR devices is the movement of liquids to and from the sensor surfaces, either mechanically or digitally by means of insulated electrodes [10].

   In 2018 Carterra released an integrated array-based SPR platform combining a 96-channel flow cell and single large flow cell which allows for the analysis of 384 immobilized or captured ligands and 48 references simultaneously in parallel for 432 total measurements per sample injection. The use of array-based SPR platforms in epitope binning enables these assays to be run at much higher scale and complexity than possible with

non-array-based biosensors. Having the ability to inject a single small volume of Ag and then competitor mAb over many ligand mAbs together drastically reduces the assay time, setup complexity, and materials required to create large pairwise competition matrices.

*Bio-Layer Interferometry (BLI):* In the early 2000s, another optically based biosensing technology, termed BLI, became commercially available. BLI relies on the phase shift for reflected patterns of light from a sensor tip, effectively measuring optical length of a fiber rather than mass on the surface [11]. Protein bound to the sensor tip changes the surface thickness and the subsequent phase shift can be detected. BLI signals require clear changes to the optical thickness on the sensor surface. This can pose challenges for assays like classical sandwich where interactions stack up and the orientation and other optical properties of the molecules are varied and impact the signal, preventing clear mass-based stoichiometry of binding. Atypical behaviors like low sandwiching signals and inverted binding sensorgrams occur as a result.

Instead of microfluidics, with BLI the samples and assay buffer reside in well plates, and the sensor tips are immersed in these solutions during binding assay steps. The 96- and 384-well plates used to hold these solutions are mixed orbitally to simulate flow and aid in transport of molecules to the sensor tip surface. Commercially available sensor tip configurations range from as little as 1 up to 96. Multi-sensor BLI platforms utilize one sensor per well arrangement, so there is no real-time referencing within a sample. All BLI instruments process samples in a by row or by column orientation, so for a binning matrix to be completed, the same sample needs to be added to numerous wells and reference locations requiring a dedicated tip and sample location.

*Less common technologies for epitope binning:* Other means of epitope binning include flow cytometry and Luminex as well as plate-based approaches such as ELISA and MSD (Meso Scale Discovery). While ELISA, MSD, and Luminex are ubiquitous technologies in many research groups, for competitive binning they involve labeling/use of additional detection reagents and washing steps which add complexity in assay setup and miss weak binding interactions [5]. Similarly flow cytometry assays also require labeling and necessitate development of higher-affinity, previously mapped mAbs to support de novo binning [12].

2. *Classical sandwich format:* Figure 1 illustrates the classical sandwich style competitive binning format. In this format mAbs are immobilized to surface; then Ag is introduced in the solution phase followed by a mAb also in the solution

phase. The ability of the solution phase mAb to bind to the Ag already bound to the surface-immobilized mAb dictates whether these two mAbs are competitive or not. At the end of each cycle, the Ag and solution phase mAb are removed from the surface by a process termed regeneration. Regeneration is typically done using a low or high pH solution that briefly denatures or alters the charge within the interacting domains to rapidly dissociate the mAb/Ag complex and allows the surface-bound mAb to bind Ag again in the subsequent cycle [13].

Classical sandwiching requires that Ags be monomeric, with only one binding site for each mAb. If targets are multivalent (dimers and trimers), then self-sandwiching can occur and complicate the analysis [5] and a premix approach is likely more appropriate.

To accurately account for the magnitude of sandwiching, an injection of Ag followed by assay buffer is used as a control. Without this control it is difficult to effectively characterize sandwiching among different mAbs since the Ag can have varying rates of dissociation to the surface-bound mAbs. Benefits of the classical sandwich format include avoiding the requirement of optimizing for saturating conditions as needed for premix or in-tandem. Limitations of the classical sandwich assay include the inability to be used when Ags are multivalent, as the solution phase mAb will often sandwich due to accessible epitope not bound to the surface mAb.

One interesting phenomenon which is not uncommon is to see displacement of bound Ag by a sandwiching mAb. This effect can either be very rapid, looking like an elution of bound Ag, or can be slower where a trimolecular complex clearly forms, but then dissociates more rapidly than the Ag alone from the ligand. This effect is often referred to as displacement. Rapid displacement is always considered blocking when applying cutoffs, but there can be times when a moderate increase in the speed of dissociation maintains sandwiching signals above cutoffs [14]. This behavior is straightforward to visualize in the classical sandwich format but is more subtle in premix and in-tandem formats.

*Premix format:* The premix format is well suited for multivalent Ags and relies on a mAb being surface immobilized, followed by introduction of a mixture of Ag in the presence of a molar excess of mAb (Fig. 2). Premixing the Ag with a saturating concentration of each mAb binds all available epitopes for that particular mAb. In the case of a homodimer, for example, this prevents one half of the Ag from binding the surface mAb, while the same epitope on the other half is bound to the solution phase mAb. For premix binning of a multivalent analyte to yield easily interpretable results, two criteria need to

be met to ensure Ag saturation in solution: (1) The competitive analyte mAb needs to be present in significant stoichiometric excess of the Ag, and (2) the concentration of the analyte mAb needs to be well above the $K_D$ (affinity) of the mAb/Ag interaction. High binidng site occupancy (>90%) is a necessity for the premix format and ideally solution mAb concentrations are maintained at least tenfold above their affinity relative to the Ag. If saturation is not achieved, then interactions can be incorrectly classified as sandwiching or demonstrate very poor competition. Often observing a mAb forming a sandwich with itself, or demonstrating limited competition, is an indicator of insufficient saturating conditions in the premix format.

As with the classical format, the surface is regenerated after each cycle, and inclusion of an Ag-only injection at the identical concentration of the mAb tests is used to quantify magnitude of sandwiching and blocking signals. The premix format can be more challenging to interpret than the classical sandwich format due to difficulties achieving saturating conditions, particularly since more mAb is required than in the classical format. Also, some behaviors such as displacers and affinity modulators can yield somewhat ambiguous results which look like weak blockers or weak sandwichers as the mass of the complex binding and kinetics can be modulated. Since the classical binning format uses iterative binding steps, the complex behaviors are more obvious.

If ambiguous data is generated in a premix assay such as partial blocking (weak sandwiching), additional titration experiments can be used to better elucidate the interaction. For example, if partial inhibition is seen with an analyte, a series of injections using a fixed Ag concentration and a titration of analyte mAb concentrations can be injected. If the clone is a competitive blocker, as the concentrations of competitor increase, the binding level or binding rate should trend toward zero. However, if the analyte mAb is an affinity modulator, the binding rate will plateau well above zero, demonstrating binding and saturation but with a new binding rate. These approaches are common to study the effects of allosteric modulators [15–17]. Benefits are that the premix format can work with both monovalent and multivalent Ags.

*In-tandem format (serial mAb addition):* In contrast to the classical sandwiching and premix formats that require mAbs to be immobilized, the in-tandem format instead immobilizes the Ag to the surface. Next one mAb is introduced followed by another and if the second mAb can bind, then this indicates lack of epitope competition (Fig. 3). In-tandem can be practical when immobilizing the mAbs is challenging, potentially the case with minimal antibody formats such as single domain antibodies (nanobodies or VHHs). This method is often

applied using a non-covalent Ag immobilization approach, for example, capturing via a polyhistidine (His) tag, which can be readily regenerated, simplifying assay development. It is important when applying a capture approach that the Ag capture step avoids having a surface that is reactive with the injected mAbs, such as an Fc capture or shared epitope tag. Benefits of the in-tandem approach include the ability to work with both monovalent and multivalent Ags.

There are several significant challenges of in-tandem approach which include (1) the need to saturate the surface-bound Ag with the first solution phase mAb, (2) the potential for steric issues from having the Ag immobilized or captured to the surface and with less accessible epitopes than when in solution, with the surface representing a third interaction in each analysis, and (3) complexity of interpreting signals from rapidly dissociating analytes. If the first antibody dissociates while the second is binding, complex data is generated [6]. The tandem method also has little ability to discern displacement and other modulating effects. In this analysis, similar to premix binning, high-affinity clones typically yield clear results, but lower-affinity clones may struggle and are best suited as the second Abs to elucidate competition.

3. *Types of competition: direct (orthosteric), steric, allosteric, and affinity modulated:* Before discussing specifics of competitive binning assays, it is important to layout mechanisms of competition that could be expected during a typical protein/protein interaction. The simplest and most likely explanation for an observed competitive event is a direct competition, where two mAbs are exclusive of one another through their binding domains which directly engage a shared epitope. In this scenario these two mAbs share significant overlap with their respective epitopes with no possibility of co-occupancy. In the case of steric competition, structural regions of one binder exclude another binder from accessing the Ag, but not because the same epitope is bound. Examples in this case would be observed competition of two mAbs which is mediated by the Fc or constant region of one mAb blocking or inhibiting access to the binding site of the other mAb. Steric interactions can be strong, inducing a near total blockade of the second binding interaction, or they can be partial, either slowing the rate or level of the second binding interaction [18].

Allosteric competition can arise through a conformational action where, for example, binding of one mAb to its epitope causes a conformational change in the Ag such that a second mAb's epitope becomes inaccessible. While described here as competitive in nature, allosteric modulation can be positive as well, making available epitope(s) normally not present on the

Ag. In contrast to the steric mechanism, mAbs engaged in allosteric competition do not directly interact with each other.

The last type of competition is affinity modulation where binding of one mAb changes the kinetic binding properties of a second mAb or binding partner. Rather than full occlusion of the epitope as in allostery, kinetic modulation changes the kinetics of a mAb toward an epitope. In epitope binning studies, most commonly this is observed as a reduction in affinity. However, kinetic modulation can be both a positive or negative modulator of affinity and result from several mechanisms including allosteric (conformational) effects, steric interference, and creation of neoepitopes [15–17].

It should be noted that in the aforementioned examples of competition, no single biophysical method alone is sufficient to fully determine the type of competition observed and orthogonal data must be considered such as mapping, mutational studies, and/or structural analysis.

4. *Considerations when choosing a technology for epitope binning:* As previously mentioned, epitope binning has demonstrated the most value in recent years when executed at a scale that can influence decision-making steps and speed up drug discovery [4–6]. Commonly this is early in the mAb discovery process and this context therefore is a helpful way to evaluate the practicality of different technologies.

   *Sensor surface configuration:* Given that early mAb candidate pools can be in the hundreds to even thousands of unique clones, throughput is an important consideration when deciding on a methodology for epitope binning. Four- and eight-channel biosensors can take weeks to perform a fully pairwise binning analysis for 96 clones. Although they can parallelize up to eight injections per cycle, this still requires new surfaces to be created eventually for remaining clones in the panel. The 96-tip BLI devices have more sensors that can be read in parallel but require that the samples are heavily replicated and reordered across multiple plates in order to test all the pairwise combinations. Therefore, the practicality of these 96 tips is complicated by the objectives of pairwise competitive epitope binning where many distinct combinations must be detected.

   *Parallel vs. multiplex:* For competitive epitope binning, multiplexing allows the testing of distinct combinations in a more rapid fashion than by a parallel approach. With a parallel approach, many combinations are tested simultaneously, but then new combinations must be prepared, often in the form of rebuilding the surfaces, which can add significant time and complexity to the assay. In contrast, multiplexing using array-based SPR can be conducted by injecting an Ag followed by a mAb across an array of 384 unique mAbs. With this

configuration competitive outcomes are determined simultaneously in 1 cycle for 384 interactions, and the complexity of the assay scales linearly, with a single additional injection per clone added to the matrix, rather than requiring a retest of that clone to all the other interactions via separate injections/dips, resulting in an exponential scaling problem as the assay size grows.

*Reagent efficiency:* The beauty of competitive epitope binning on biosensors is that it only requires mAbs and their respective Ag. No additional binding reagents are needed for a basic competitive binning exercise. However, the mAbs and Ag are often in finite supply depending on (1) the scale the mAbs were prepared at, which at an early stage are often in low microgram quantities, and (2) whether Ag can be expressed and purified in house vs. needing to purchase commercially. Assays typically need to be conducted that consume microgram amounts of mAb and Ag, rather than milligram amounts. Having a high number of unique sensor surfaces that can be multiplexed is the most efficient way of conducting competitive epitope binning in terms of reagent usage.

*Crude vs. purified:* Where possible it is desirable to avoid purification steps for crude samples such as hybridoma supernatants, given the additional time and resources required for clones that are yet to be proven as viable candidates. Challenges with working with crude sources include highly variable expression levels and the potential presence of confounding components. Since SPR- and BLI-based epitope binning protocols use analyte mAb concentration to drive binding events, some crude sample sources such as small-scale bacterial extracts or B-cell supernatants may have insufficient mAb concentrations for use as analytes (the on-rate and affinity of the interaction determine the molar concentration required). Many sample types like exhausted clonal hybridoma supernatants or mammalian transient expression systems (HEK293 and CHO) often contain sufficient mAb levels to work well in binning assays. The other main challenge with crude samples is the need to immobilize the mAbs to the sensor surface. If the mAb is not the dominant protein species in the sample, then it likely cannot be directly coupled to the chip surface and capture then cross-link-based approaches are necessary.

As mentioned previously, for the scale often needed for screening early-stage clones, the configuration of BLI and traditional two- to eight-channel SPR instruments are less desirable in terms of throughput and sample consumption and are impractical for characterization of large panels. For SPR-based biosensors, there can also be a risk of clogging on certain systems with very narrow flow path dimensions of the microfluidics, although filtering of the samples using 0.2 μm

filters will largely eliminate the issue. High-throughput SPR (HT-SPR) instruments, such as the Carterra LSA and LSA$^{XT}$, are designed with larger microfluidic paths unlikely to be affected by crude sources. In terms of signal complications arising from crude sources, usually this can be accounted for by a combination of sufficient sample dilution into assay buffer as well as monitoring of responses immediately after the injection of crude material has occurred, in which only assay buffer is flowing, and signals are clearly discernable.

*Selecting a technology:* When conducting epitope binning for greater than 32 clones, common constraints such as high clone numbers, minimal available sample, and the need to complete assay in days rather than weeks or even months means there are limited options with available technologies. Since array-based HT-SPR meets these needs, it has become a leading technology for epitope binning [19–23], and by far the most common platforms used currently in industry for HT-SPR and large-scale epitope binning are the Carterra LSA and LSA$^{XT}$. Epitope binning approaches and data analysis strategies adaptable to the LSA and LSA$^{XT}$ will be discussed here.

5. *Comparison of epitope binning to epitope mapping:* As previously described, epitope binning is used to assess whether mAbs share similar or disparate epitopes, by means of assessing whether two mAbs can bind Ag simultaneously. Competitive-based epitope binning is attractive because it measures binding directly and only requires mAbs and corresponding Ag, at least when deployed on label-free biosensors. However, without inclusion of binders to known regions of the Ag, it cannot provide specific domain or amino acid-level resolution of epitope binding sites. Strategies such as inclusion of controls with previously described structural biology can aid in informing and orienting these competition based epitope bins.

   In contrast, peptide-based epitope mapping is a means to identify domain and possibly amino acid-level recognition. Epitope mapping requires the use of peptides derived from the Ag, most often designed as overlapping regions and covering the entire Ag sequence. mAbs are tested for binding against each peptide and through recognition of a specific peptide or peptides, mAbs binding domains can be assessed along the antigen sequence. It is important to note that only linear epitopes are recognized using peptide-based epitope mapping. While epitope mapping provides localization of epitope that is typically not achievable in competitive binning, some conformational epitopes may not be recognized. It is possible to conduct epitope analysis using both methods and understand epitope relationships among mAbs (competitive binning) in conjunction with epitope localization (epitope mapping).

There are growing public databases of published binding epitopes and structures which can be used to identify controls and inform analysis (www.IEDB.org). In addition to peptide-based epitope mapping, array SPR can be used to create arrays of mutant proteins, such as alanine scan approaches. mAbs can be evaluated for binding to libraries of mutants in much the same way as for the peptide approach described above.

6. *Immobilization considerations:* When designing a competitive epitope binning assay using HT-SPR, two of the most critical factors are effective immobilization of surface-bound mAbs and robust regeneration of the surface between cycles. For some clones with minimal antibody formats such as VHHs, direct amine coupling may be challenging as it can impact activity. In these cases, strategies such as capture-crosslinking or minimal biotinylation can provide opportunities for more directed immobilization.

   For purified mAbs the simplest approach is to activate carboxymethyl groups to N-hydroxysuccinimide esters on the chip surface using a mixture of EDC and sNHS, typically at 100 mM and 25 mM, respectively. This surface then becomes reactive toward amine groups on the N-termini and lysine side chains of the mAbs, forming covalent bonds to the amines. To enable immobilization, the mAbs are prepared in a low ionic strength buffer that is below their isoelectric point (pI) so that they electrostatically attract to the negatively charged sensor surface (termed preconcentration) and are then capable of reacting with the free esters. Typically, this low pH buffer is 10 mM sodium acetate, with pH range of 4.0–5.5. Higher pH generally fails to establish an overall net positive charge to the protein and too low a pH can cause denaturation and coupling inefficiency. To inactivate remaining NHS esters, 0.5–1 M ethanolamine or similar free amine is injected as a final quenching step.

   To ensure robust coupling and optimize immobilization levels, preconcentration injections of ligands can be run over nonactivated surfaces. Electrostatic ligand preconcentration can be verified and optimized using different pH or mAb concentrations. The preconcentrated mAbs are easily washed away using a neutral or higher pH solution with physiological salt concentrations such as HEPES buffered saline. Some molecules may stick to the chip matrix, and using a buffer with an additional 150 mM NaCl (300 mM total) will typically result in removal of the preconcentrated protein. Note that electrostatic preconcentration is only efficient in samples with low total ionic strength. The presence of salt or other ion concentrations exceeding 25–30 mM will show markedly reduced electrostatic preconcentration and failure to couple. At physiological salt concentrations, most mAbs will show no electrostatic

preconcentration even at pH 4.0. Ensure that ligands are diluted sufficiently into the 10 mM sodium acetate to achieve these low ion concentrations. While immunoglobulins (IgGs) typically preconcentrate very well at pH values between 4.5 and 5.0, smaller mAb fragments like VHH and scFvs can have more diverse pI values and behavior. Running a scouting is recommended for these constructs; using an acetate solution on the lower end of the pH range such as 4.25 for immobilization is often an effective strategy.

The concentrations of mAbs used as ligands are typically between 0.1 and 15 ug/mL. For epitope binning, higher binding signals are usually easier to interpret and so there is limited value in targeting the low ligand densities preferred in kinetics analysis. Appropriate ligand mAb immobilization levels are commonly between 500 and 2000 RU or more and depend on final fractional activity of both the ligand and Ag as well as overall molecular weight of the Ag.

7. *Binning using crude samples:* mAbs from crude samples such as hybridoma supernatants can be characterized by epitope binning, but there are a few additional steps required to correctly prepare the surface for the experiment. By starting with a capture surface, such as an anti-IgG Fc polyclonal antibody, the mAbs can be captured out of solution and effectively enriched on the chip surface, while other components of the sample are washed away. The fluidics of the LSA allow for bidirectional flow of the samples which aids in enriching mAbs onto the capture surface under high flow for extended contact time, allowing for enrichment of low concentration samples. This bidirectional flow can be run for up to 1 h and consumes no additional sample in the process.

   Since regeneration is needed after each cycle in the binning experiment, the captured mAbs are then covalently attached to the sensor surface. This is best done after capturing the ligands by injecting a crosslinking reagent such as bis (sulfosuccinimidyl)suberate (BS3), a commercially available homobifunctional water-soluble amine-to-amine crosslinker [24]. After crosslinking, the surface is quenched with an injection of ethanolamine to remove any remaining active esters.

   As the surface can still bind free IgG Fc, blocking is required in each cycle. By selecting the blocking option in the method, a solution of irrelevant IgG is injected for 5–7 min time to ensure the solution phase mAbs do not react with the surface. Concentrations of blocking IgG will need to be determined empirically, but 200 μg/mL is typically sufficient. A control mAb of the same species but does not recognize the Ag can be incorporated into the experiment as an injection to confirm the surface is not reactive following the blocking step,

and similarly monitoring binding to reference surfaces can also be used to gauge blocking efficiency.

8. *Ag and regeneration optimization:* Appropriate Ag concentration enables a clearly discernable signal for mAb sandwiching above the system background, without using more Ag than necessary. Typically, this ranges from 25 to 100 nM. This is easily determined by preparing the Ag at a few concentrations and injecting to confirm appropriate responses. Ideally Ag response levels greater than 20 RU are required.

   In the classical sandwiching format, the concentration of Ag is less crucial, and the process of optimization of Ag concentration is often focused on reducing sample consumption more than increasing assay performance. For example, if sufficient binding is seen using 50 nM Ag, then using 500 nM would likely not negatively impact the data quality but would use 10× as much Ag. The Ag concentration does not impact analyte mAb requirements assuming overall response conditions are met. For the premix format however, the analyte mAb must be in molar excess relative to the Ag, so using the minimal Ag necessary for robust signals should be selected, as higher Ag concentrations require more analyte mAb to achieve saturation. Therefore, in premix assays, optimizing for the minimal amount of Ag necessary is a valuable step in reducing subsequent quantities of mAb required in each competitive cycle and increasing the chances of achieving robust blocking.

   Regeneration of the sensor surface after each cycle in a competitive binning experiment is critical to achieving reliable and high-quality data. Fortunately, the classical and premix formats utilize mAbs immobilized on the sensor surface, and as a class mAbs are generally acid tolerant and remain active after brief exposure to modestly denaturing conditions. Following immobilization of mAbs and often as part of the Ag optimization process, conditions need to be found which fully remove bound Ag from the surface but still allow for nearly identical Ag binding levels in subsequent cycles.

   Typical regeneration conditions rely on low pH ($<3$) to release bound Ag. Alternatively high pH ($>9$) can also be used in cases where low pH proves ineffective. Very harsh pH conditions ($<1.7$, $>12$) tend to cause irreversible denaturation of many mAbs. Addition of salts and/or detergents can also be used or added to remove bound Ag if pH alone is ineffective. Table 1 lists common types of regeneration solutions used in HT-SPR assays. Commonly, a minimum of three to five cycles are needed to evaluate the performance of a regeneration condition, with the baseline following regeneration and the magnitude of the Ag response in the next cycle confirming (1) the removal of bound Ag and (2) the preservation of ligand activity. Since incorrect regeneration can result in either buildup of Ag

on the surface or by inactivation of the ligand, tracking both baseline levels and Ag response will elucidate the behavior. After immobilization it is typical that some variance in baseline and binding response will be seen over the first few regeneration cycles, so more than three cycles are often required to demonstrate reproducible binding from a new surface.

9. *Use of controls:* Controls are important in competitive binning assays for several reasons. As already mentioned, the activity of ligand mAbs during a run can be affected by regeneration conditions, and this can differentially impact certain mAbs compared to others. By inclusion of regularly spaced Ag-only injections, any changes in ligand mAb activity are easy to monitor and account for during data analysis. Furthermore, the inclusion on the sensor surface of molecules that react with the Fc region or expression tag of the mAbs can help confirm that the sample did in fact pass across the surface at the expected concentration. This is helpful when a mAb fails to show any sandwiching activity across the array and confirms there was not a sample preparation issue. Likewise, a ligand reactive with tags found on the Ag is helpful for the same reason, anti-His being an example, as all active analytes which bind the antigen outside the tagged region should show sandwiching signals. Negative controls or Fc binders can be included for reference but will need to be excluded from the final processed data set, as only Ag binding ligands and analytes should be included in the final epitope binning analysis.

   While not essential to conduct a competitive epitope binning assay, inclusion of benchmarks such as mAbs with published structural data or landmark reagents such as receptors that bind to a known epitope of the Ag help to guide interpretation of binning outcomes. Benchmarks can be a single mAb or even multiple mAbs to several epitopes used as a tool to guide selection and optimization and navigate intellectual property concerns. Landmark reagents can be valuable to localize mAb clusters to sites involved in binding pathways and to focus on competitive mAbs.

10. *Data processing:* Referencing is used to subtract injection artifacts, such as buffer refractive index changes, from the active surface. By selecting the reference option in the Epitope software, a signal from a surface which is inert to the analytes in the assay is subtracted from an adjacent active surface. On the LSA the signal for all 48 references can be viewed separately in an array view (Fig. 5a). The data can be viewed before and after reference subtraction (Fig. 5b), showing removing of the square-shaped refractive index shift as the solutions change.

    Cropping is used to remove portions of data not needed for final data analysis, such as the regeneration injections. While not necessary, it does simplify the data view and makes interpretation easier. An example is shown in Fig. 6.

Y-align is used to adjust sensorgram signals at a defined x-scale location to zero on the y-scale. This is helpful both to standardize the magnitude of signals evaluated and also to look for overall trends in baseline across the experiment which can be indicative of sub-optimal regeneration. Figure 7a highlights a clone in pink using a serial y-alignment approach where a clear shift in baseline is occurring because of incomplete regeneration of certain sandwiching pairs. Figure 7b shows how a global y-alignment can be performed in overlay view so each cycle starts at zero. This step is required before applying binding cutoffs and interpreting binning results. Figure 7c shows a data set in serial view after the global y-alignment has been applied.

11. *Normalization and measurement bars:* In Carterra's Epitope analysis software, the Normalization bar is used to scale the sensorgram response levels and account for decreased Ag binding during the experiment. The y-value of the sensorgram near the maximum Ag response is used to divide all other y-values across the sensorgram. The result is that this position of Normalization bar (maximum Ag response) becomes 1 on an arbitrary response scale and all other values across the sensorgram adjust proportionally. For classical sandwich this normalization is simple, setting all Ag binding levels to 1.0 for all cycles (green bar in Fig. 8a). For premix a different approach is used where Ag alone controls are spaced evenly throughout the assay and scaled to a value of 1.0. Then the software looks at the slope or change in Ag binding between the various control injections and scales the experimental sensorgrams accordingly. It is important that the Ag alone controls be well behaved, evenly spaced, and an identical concentration to the analyte test samples for this normalization to apply appropriately. Viewing the raw non-normalized premix data can be a good tool to troubleshoot any complexity seen in the normalized data. The position of this normalization bar in premix binning is also the report point location for the analytes and used to set cutoffs for blocking and sandwiching.

    The Measurement bar is specific for analysis of classical binning experiments and determines the magnitude of sandwiching relative to the Ag-only buffer injections (orange bar, Fig. 8a). This is because in classical experiments the sandwiching response occurs after the initial Ag injection and while Ag is dissociating from the surface. Using the difference between the Ag-only response levels and the Ag followed by mAb response levels allows for a highly accurate determination of sandwiching magnitude even for ligands with fairly rapidly dissociating Ag (Fig. 8b, c). There is a limit where if Ag dissociation from a ligand is too fast, it can be hard to interpret sandwiching signals, especially with monovalent analyte mAbs. With IgGs

or other bivalent analytes, sandwiching tends to have a massively stabilizing effect on Ag dissociation, reducing the apparent rate of Ag dissociation by more than an order of magnitude and making the sandwiching responses obvious. With monovalent analytes such as scFv and VHH binders, the stabilization effect is typically much weaker or not present at all, so short analyte injections (e.g., 1 min) may allow for more accurate determination of rapid systems.

12. *QuickClean settings:* See Fig. 9 for illustrations. Ligand mAbs that fail to bind Ag need to be removed prior to data analysis. The Epitope software enables this by selecting the "Ag capture less than option" and then choosing "Disable Highlighted." If many cycles for a given ligand are removed, it suggests a poorly active surface, and the "Ligands with more than 5 Excluded Experiments" option can be chosen to remove an entire ligand. Alternatively, the ligand behavior can be viewed in the Ligand Array view, and clones with insufficient binding can be recorded and removed from the main ligand table on the main data page. This approach gives the user a clear view of what is being removed and the overall ligand performance.

    mAbs that fail to self-block can also be highlighted using the "Ligand does not self-block option" and investigated further. Failure to compete can sometimes be a result of background effects and simply require a slight adjustment of the threshold to remedy. High self-sandwiching is a sign of possible misidentification of a ligand or analyte, a mixed clone, a complex or nonspecific binding behavior, etc. Self-sandwiching that is widespread may suggest a multivalent and/or aggregated Ag. This is sometimes seen when a monovalent Ag has dimerized via its expression tag.

13. *Analyte curation:* Like with ligands, only active analytes should be included in the final binning interpretation. For premix assays, inactive analytes tend to be flagged as sandwichers (no blocking at all) and should also be excluded (Fig. 10a). In classical sandwich assays, inactive analytes will typically appear to have a blocking relationship (no sandwiching) for all ligands and need to be excluded (Fig. 10b). Clones which universally sandwich, even with themselves, should also be excluded and investigated for root cause such as possible lack of clonality.

14. *Competition threshold:* The competition threshold is a key part of data analysis for classification of sandwichers, blockers, and intermediates. The competition threshold is defined by a horizontal bar on the y-scale of the sensorgram plot and can be set either globally based on the normalized binding level for all ligands or adjusted on a per ligand basis (Fig. 11). Intermediate binding responses are captured by adjusting the thickness of threshold bar. These intermediates are displayed as yellow in

the heat map to visually aid in curation but are considered as sandwichers in the binary assessment of sandwiching (green) vs. blocking (red).

There is no clearly established mechanism to determine an ideal threshold for the cutoffs. Well-optimized assays with strong binders and sufficient mAb concentrations often result in assays where simple global cutoffs completely capture the assay result. Other times there are often layers of complexity within the data which need to be addressed. One school of thought is that it is best to take a rigid approach of using fixed global cutoffs, even between experiments. The thought is that this eliminates analysis bias in the interpretation of the data and leads to consistent processing from assay to assay. Both points are certainly true, but for binning maps, epitope bins, and community groups to be most meaningful and accurately depict epitope space, applying a more scrutinizing and deliberate approach can have advantages. For example, a tight global cutoff of 0.25–0.28 could be set in a classical sandwich assay that puts 95% of clones in the correct assignment with only a few yellows. When sorting the heat map, it may be evident that a few points for a handful of ligands seem to defy otherwise discrete behavior of the binning map or even that a clone is marked as a self-sandwicher. By looking at the sensorgrams, it may be observed that one clone sandwiched weakly generally, likely due to low affinity and/or lower than ideal analyte concentration. Notice that the clone looks to have a binding signal that is just below the cutoff for the apparent outlier ligand or a few ligands and just above the cutoff for many others. It also displays clear blocking with no binding for other clones consistent with it fitting into a bin group. In this case manually lowering the cutoff for the ligands with the "just below the cutoff" behavior to mark them as sandwichers allows the clone to cluster neatly into a bin group and improves the overall clarity and interpretability of the analysis without impacting very many if any other calls in the matrix.

Another common example is an assay with generally good behavior but one or a couple of clones show a small level of self-sandwiching. When investigating these clones, which show low self-sandwiching signals just above the cutoff and they also show clear sandwiching and blocking relationships within the ligand and as analytes sandwich and block strongly to other ligands. Adjusting the cutoff to make this low self-versus-self interaction set as blocking, often with a few other analytes with nearly identical behavior also being reclassified, can significantly simplify the heat map and the bin clustering. A few conditions can result in this type of low-level self-sandwiching, but a common reason is that the Ag may have a moderate level of aggregate or dimer. Some clones bind readily to epitopes presented by the dimer and can show moderate levels of self-

sandwiching, and other clones bind to epitopes obscured in dimers or aggregates and show no self-sandwiching as they in fact do not bind the aggregated Ag at all. When applied conscientiously, these common cases of the need to manually adjust cutoffs and accommodate the behavior of specific ligands and analytes can significantly simplify the presentation and interpretation of binning analysis and more accurately reflect the biophysical reality of the system being analyzed, as long as they are applied carefully and thoughtfully to generally high-quality data.

Assays with many low activity analytes, poor regeneration, inconsistent Ag binding, and/or compromised Ag (dimerized, aggregated, clipped, etc.) can often not be saved by extensive customization of cutoffs, and the interpretation of the results does become subjective. In this case rigorously excluding low performing analytes and ligands and only interpreting the clear data is an appropriate approach, or ideally performing additional assay optimization and repeating the assay under more optimized conditions to yield clearer data.

Setting cutoffs in classical sandwich assays is often simpler than premix binning assays. In a classical sandwich assay, any level of sandwiching is discernable from the control, so the nature of the sandwiching response (tight, weak, slow, fast, low, high, etc.) is clearly visible. In the premix assay using a multivalent analyte, incomplete saturation can look like a weak blocker or affinity modulator. If saturation is not achieved, responses looking similar to the Ag alone can be achieved. Again, looking at the clone performance in other contexts (as a ligand and as analyte against other ligands) can inform the behaviors for individual clones and can inform the decision for cutoff setting. For premix, interpreting a partial blocking signal in the absence of competitor dose response can be a challenge. For premix binning using high analyte mAb concentrations which drive saturation simplifies the binning results. Typically for IgGs 50 μg/mL (333 nM) is a reasonable target. High-affinity systems can get away with lower analyte concentrations, but even at 333 nM, panels with triple digit nM affinities will not be fully saturated regardless of stoichiometric excess and it may be possible to include even higher analyte mAb concentrations

15. *Asymmetry:* An additional feature that is helpful in heat map curation in the Epitope software is the Asymmetry highlighting option. This allows the user to highlight competitive events that do not recapitulate when the analyte and ligand mAbs are reversed, termed asymmetric (Fig. 12). While asymmetry can be mechanistically feasible due to steric or allosteric phenomena, the threshold settings should first be investigated, particularly if intermediate sandwiching responses are present. It is

common that small adjustments to the cutoffs can eliminate many asymmetries, greatly simplifying the interpretation. If asymmetries are verified to be correctly assigned, then they can remain in the analysis. Asymmetries are reflected in a network plot by a dotted competition chord.

*Allostery and kinetic modulation:* Several complex binning behaviors can arise given the flexibility and complexity of biomolecules and their binding properties. A common one is displacement or when a ternary complex, once formed, induces a much more rapid dissociation of Ag from the ligand than it would normally have. In Fig. 13 there are examples of a mAb ligand showing very rapid dissociation of Ag in the presence of mAb analyte as well as an example where the dissociation is faster than normal but still allows for a significant buildup of ternary complex to be seen and then a dissociation slope during the analyte injection. These interactions are typically treated as blockers, although some with modest rates of displacements can still fall within the sandwicher cutoff range.

16. *Heat map:* The Epitope software heat map depicts the competition matrix with ligands as rows and analytes as columns (Fig. 14). The values present in the normalized heat map are the difference relative to control at the report point position. Green cells show sandwiching, red cells show blocking, and yellow cells show intermediate activity. The self-versus-self interactions are shown as dark outlined cells. The heat map can be sorted to bring similar clusters together. Sorting can be performed iteratively while the data cutoffs are adjusted and the ligand/analytes curated to remove poor performers.

    When the heat map is sorted, a dendrogram is created. These dendrograms depict the similarity distances between the competition profiles of the clones with emphasis on shared blocking relationships. Two commonly applied algorithms for this type of exercise are McQuitty and Ward.D2.

17. *Dendrogram and network plots:* In the Epitope software, a network plot displays every clone as a node, either as a circle (clones present in both the ligand and analyte direction) or a square (clones present as only a ligand or analyte). A chord connecting two nodes means that the clones are competitive with each other. Dashed chords mean that the competition between the clones is asymmetrical, with a blocking relationship in one orientation and sandwiching in the other. Clones with identical competition profiles within the heat map are shown as the same color and contained within a shaded colored region known as an epitope bin. Note that in the network plot, the relationships shown are binary and a clone is either a blocker or a sandwicher (or asymmetrical which is one of each). This means that the binning map can be manipulated

to enhance visualization by changing the position and colors of bin groups without affecting the meaning of the plot. The default position algorithms attempt to position things close to the clones with related profiles and separate more distantly related ones, but some manual manipulation is often applied to aid in the interpretation of the results.

As mentioned above the sorting algorithm creates a dendrogram where clones with similar competition profiles are grouped together on branches. At the bottom of the dendrogram, there are horizontal lines representing the epitope bins or clones with identical competition profiles. As the branches move up, there are more and more differences among the clones in their competition profiles. In large binning assays with high epitopic diversity, there can be many closely related branches. As the branches go higher, they become less related, eventually converging at the top even if they have no relation. Typically, the most meaningful associations are low on the dendrogram, but sometimes mid-level branches represent meaningful clustering schemes like binding to an inside versus outside face of a protein. There may be a lot of meaningful differentiation within the lower subclusters, but the higher levels can still represent some meaningful biophysical reality.

Given the fine resolution and possibility for high diversity, it is often appropriate to describe and associate closely related clones with highly overlapping epitopes as clusters referred to as communities. Communities are defined by manually selecting a common height on the dendrogram and then selecting the option to create a community plot. This creates a new version of the network plot depicting the community clusters. These communities can be used to color the axes of the heat map.

Since the selection of the cut height on the dendrogram for the creation of the community is arbitrary, careful consideration of the cut height should be made. Here we propose the consideration of three simple rules for proper community assessment.

(a) Clones within a community should block the other members of the community.

   (i) The point of the community is to cluster clones which share core overlapping and related binding epitopes. If clones within a community sandwich with each other, it means they bind to nonoverlapping epitopes and the community is overgeneralized for most purposes.

(b) Clones within a community should have generally consistent behavior with regard to clones in other communities.

   (i) In a sense it should be simple to describe the behavior of communities and how they relate to one another.

For example, being able to say the clones in red community generally sandwich with clones in green, blue, and orange communities and block clones in purple and blue/green communities would be appropriate (Fig. 15). Less ideal are descriptions like the red community (Fig. 16) with clones M and N blocking the blue community and sandwiching with the green community while the rest of the red community clones have the opposite relationship with these two communities. This complexity in relationship between the bin clusters suggests that there are shared and differentiating behaviors among clones within a community. Finding a lower cut height which splits these groups into their more discrete behaviors is recommended and would improve the value of the interpretation of the clustering for most purposes relating to clone behavior and mechanism of action (MOA).

(c) It is acceptable if some communities contain complex profiles like those described in rule #2, but these behaviors should be carefully documented. These can be best described as discrete subsets as subclusters like in the red community.

(i) Complexities in dendrogram clustering often arise from communities being made up of very different numbers of clones and with widely divergent numbers of blocking relationships. Take an example of a 200-clone binning set. One community has 80 highly related clones in it that bind to a common overlapping epitope and have many shared blocking relationships within the experiment. If clones in this large cluster with lots of competition show only a few, say 3 or 4 blocking differences within a set of 130 competitive relationships, it would be interpreted as a modest difference by the dendrogram. Another group of clones may bind to a rare epitope cluster which sandwiches with the vast majority of clones in the heat map and has relatively limited number of blocking relationships, say five to ten total depending on the clone in the cluster. If these clones share several blocking relationships within the set and differ by only a few, they will be clustered at a similar point in the dendrogram as the clones from the richly sampled group due to a similar number of differences. Hower, given the small number of total competitive interactions this cluster, the small number of differences is likely more meaningful or can represent more significant differences within this group than the larger bin. By moving the

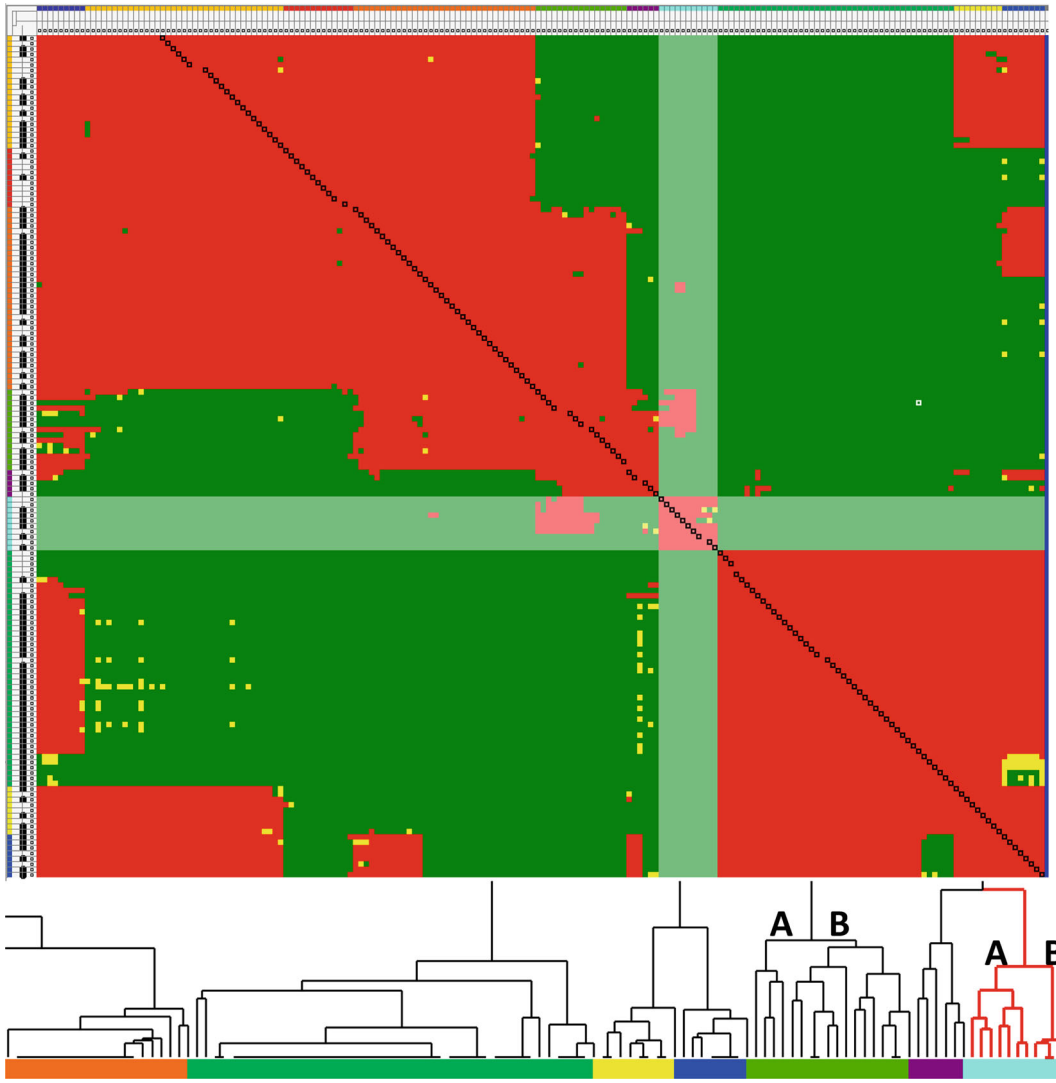


**Fig. 17** Community and subsets. The blue group (community 7) is small but complex. The A–B branching of the light blue cluster differentiates a subset of clones (A) with competition against a subset of Community 5 (A) of the green group. It is challenging to place a cutoff at a level which preserves some other large bin clusters, so the in this example the cluster is set to keep these as a single cluster, but these distinctions should be clearly noted when the data is reported and referred to as subsets A and B withing the community

cutoff down to capture these differences, larger more seemingly coherent clusters may be broken apart. In the example in Fig. 17, a small cluster light blue has two main branches ("I" and "II"), one of which competes with some of the clones of the smaller green bin. In this large assay, the clusters become heavily fractured if the cutoff is lowered, but key subclusters can be easily called out and described. Therefore, an approach which allows for higher-level clustering for visualization and organization purposes while calling

out meaningful differences within communities is a practical strategy [21]. For example, in the dendrogram in Figure 17, there are 7 main clusters, but in reporting two of the clusters would be described as subsets- cluster 5A, 5C, 7A, 7B. It is important when describing and reporting Epitope binning data that the relevant differences between competition profiles are clearly reported. Epitope competition data is most valuable when the competition data is clearly defined, with good experimental methods, careful data processing and curation, and summarized in such a way as to capture and represent accurately the diversity of competition behaviors present in the set. Over clustering of the dendrogram reduces the utility of these assignments and should be generally avoided.

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