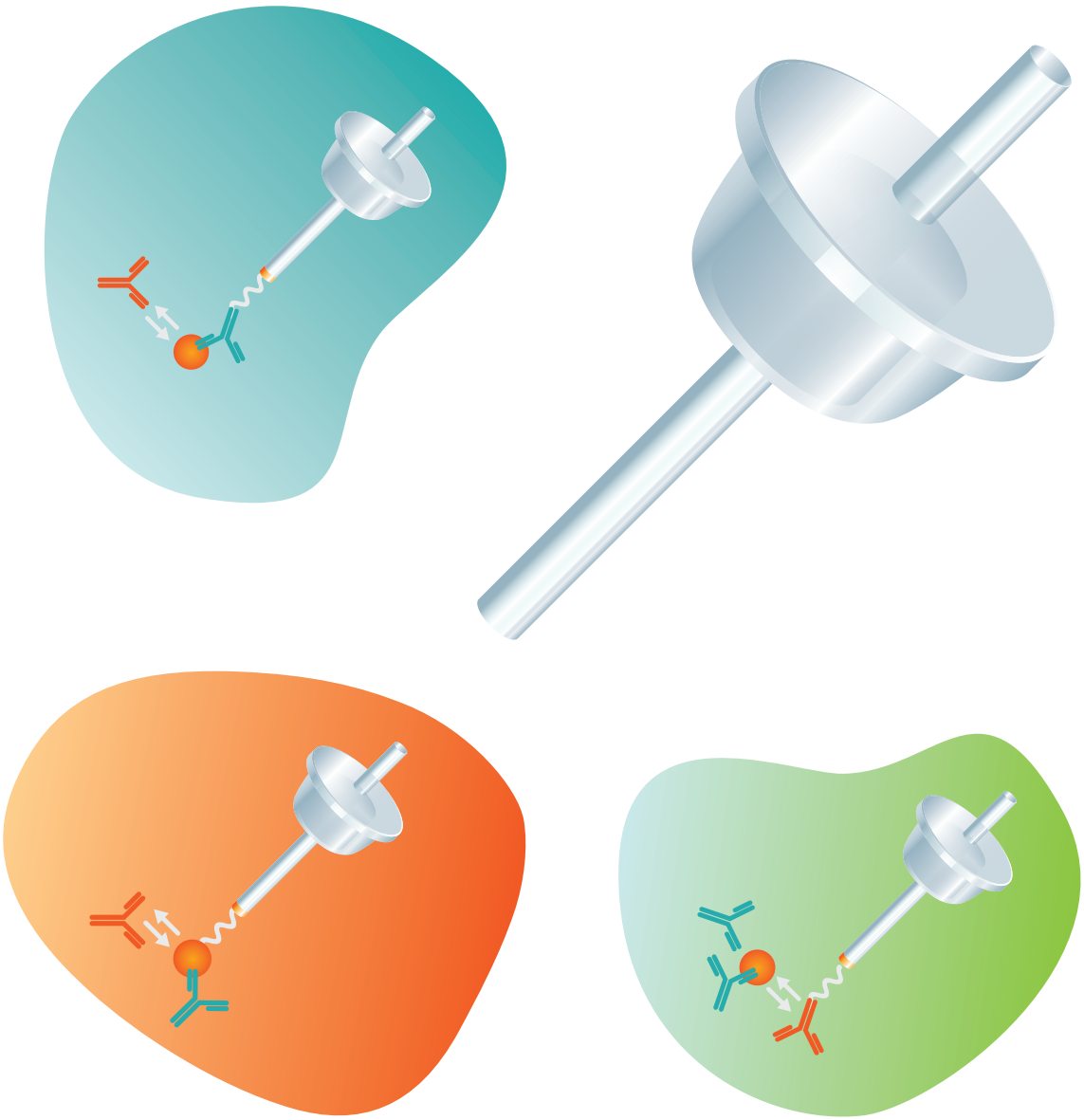
gator

Application Note



Epitope Binning Master Class

A Comprehensive Guide

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Part 1: Introduction

What is Epitope Binning?

Epitope binning is a vital process used in immunology and biochemistry to classify antibodies based on their binding specificity to distinct epitopes. Epitopes are specific regions of antigens that antibodies identify and bind to. This process holds significant importance in antibody characterization and plays a pivotal role in various domains, including diagnostics, therapeutics, and vaccine development.

Traditionally, epitope binning techniques rely on competitive binding assays, where antibodies are assessed for their ability to inhibit the binding of other antibodies to a particular antigen. These assays often involve labeling antibodies with different tags or using other detection methods to differentiate their binding.

However, an emerging alternative to traditional epitope binning is label-free technology, which directly measures binding interactions between molecules without relying on traditional labels or tags. Methods like surface plasmon resonance (SPR) or biolayer interferometry (BLI) can be used to monitor binding events between antibodies and antigens.

Label-Free Epitope Binning Assays

Epitope binning using label-free technology involves immobilizing a panel of antibodies on a sensor surface, such as a sensor chip in SPR or a biosensor tip in BLI. The antigen of interest is then introduced, and real-time measurements capture the binding interactions between the antibodies and the antigen. These binding profiles and kinetic data allow for the categorization of antibodies into different epitope bins based on their binding patterns.

One key advantage of this approach is its label-free nature, eliminating the need for additional reagents or antibody modifications. This simplifies the experimental workflow, reducing potential artifacts from labeling procedures. Additionally, label-free technology enables the simultaneous analysis of multiple antibodies, facilitating the efficient characterization of large antibody panels.

Overall, label-free epitope binning provides a powerful tool for understanding antibody-antigen interactions, aiding in the selection and development of antibodies for various applications like diagnostics, therapeutics, and vaccine design. Its label-free nature and high-throughput capabilities make it invaluable in antibody engineering and development pipelines. By providing real-time kinetic information and enabling the analysis of multiple antibodies simultaneously, label-free epitope binning advances antibody-based research and applications in diverse fields.

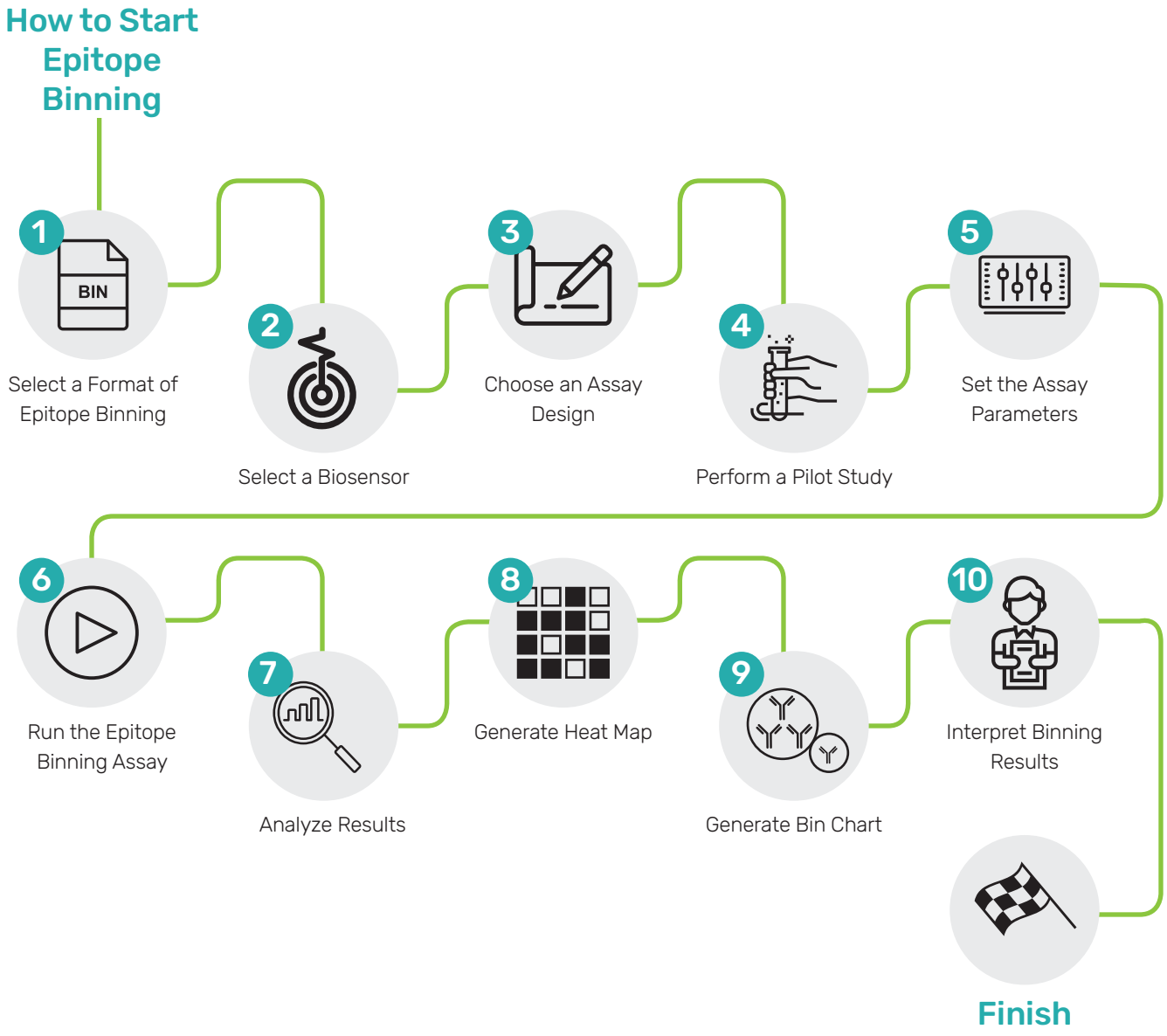
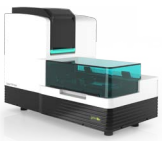


Figure 1. Flow chart for epitope binning assays.

Gator® BLI Instruments

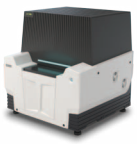
The Gator® instrument portfolio offers a diverse range of multi-functional instrument platforms based on Biolayer Interferometry (BLI). BLI is a label-free technology designed for real-time measurement of molecular interactions, serving the purposes of detection, quantitation, and kinetic analysis. The Gator system employs a standard microplate format, allowing automated high-throughput binding analysis in both 96-well and 384-well microplates. It requires minimal instrument maintenance and offers versatile assay design capabilities, ensuring efficient experimentation for researchers. Different instrument options are available to accommodate various sensitivity and throughput requirements.

Gator® Pilot



Gator® Prime

Gator® Plus



Gator® Pivot

Gator® Pro

Performance					
Type of analysis	Proteins, antibodies, peptides, nucleic acids, liposomes, viruses, small molecules				
Simultaneous reads	4	8	8	8 and 16	8, 16, 24, and 32
Maximum sample capacity	40	168	456	816	1152
Molecular weight	> 150 Da				
Association rate (k_{on})	10^1 to 10^7 $M^{-1}s^{-1}$				
Dissociation rate (k_{off})	10^{-6} to 10^{-1} s^{-1}				
Affinity constant (K_D)	10 pM – 1 mM				
Quantitation range (Protein A biosensor)	0.02 – 2000 µg/mL				
Binning capacity	6x6	12x12	16x16	20x20	32x32
Baseline noise (RMS)	≤ 4 pm				
Baseline drift	≤ 0.12 nm/hour	≤ 0.12 nm/hour	≤ 0.1 nm/hour	≤ 0.1 nm/hour	≤ 0.1 nm/hour
Acquisition rate	2, 5, and 10 Hz				
Specifications					
Spectrometers	4	8	8	16	32
Sample microplate*	96-well format[1]	96-well format[1,2]	96 or 384-well format[1,2,3,4]	2 x 96 or 384-well format[1,2,3,4]	3 x 96 or 384-well format[1,2,3,4]
Evaporation control	No	No	No	Yes	No
Sample temperature control	Ambient plus 4°C to 40°C	Ambient plus 4°C to 40°C	Ambient plus 4°C to 40°C	15°C to 40°C	Ambient plus 4°C to 40°C
Automation compatible	No	No	No	Yes	Yes
Minimum sample volume	180 µL[1]	130 µL[2]	40 µL[4]	40 µL[4]	40 µL[4]
Smart monitoring	No	No	No	Yes	Yes
Self-cleaning	No	No	No	Yes	Yes
Dimension - HxWxD (cm)	49 x 68 x 33	47 x 67 x 31	68 x 73 x 44	92 x 87 x 79	84 x 114 x 77
Weight (kg)	31 kg	35 kg	55 kg	130 kg	220 kg

*Gator Bio offers [1] 96-well flat-bottom, [2] 96-well tilt-bottom, [3] 384-well flat-bottom and [4] 384-well tilt-bottom microplates for range of BLI applications

Part 2: Types Of Epitope Binning

There are three major formats of epitope binning: in-tandem, classical sandwich, and premix. The decision chart in Figure 5 can assist in selecting the appropriate format.

In-tandem format

In this format, the antigen is first captured onto a biosensor and then presented to two types of antibodies in consecutive steps. The primary antibody should always show binding to the antigen, whereas the competing antibody will bind only if it recognizes a non-overlapping region of the antigen in comparison to the primary antibody.

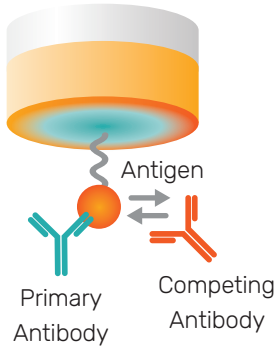


Figure 2: Epitope binning in in-tandem format.

Classical sandwich format

In this format, primary antibodies are immobilized onto a biosensor, and then the antigen is incubated with them. Subsequently, competing antibodies are introduced to form a sandwich configuration.

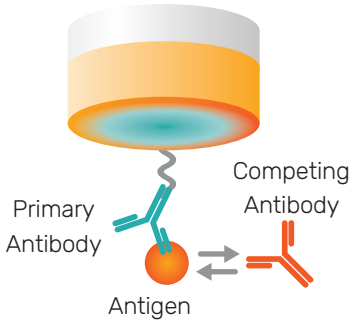


Figure 3: Epitope binning in sandwich format.

Premix format

In this method, the antigen is incubated with a large molar excess of primary antibody to create a premix sample. This premix sample is then introduced to the biosensor, which is loaded with the competing antibody. The premix format is commonly employed to resolve ambiguous responses observed with other binning formats such as sandwich and tandem. However, there are some drawbacks associated with this method, such as the need to determine dissociation constants (K_D) for antibodies in advance.

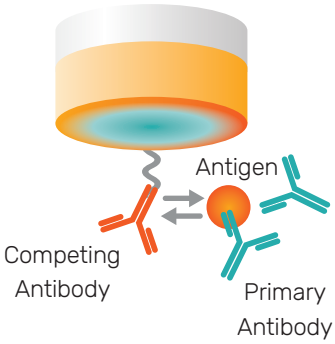


Figure 4: Epitope binning in premix format.

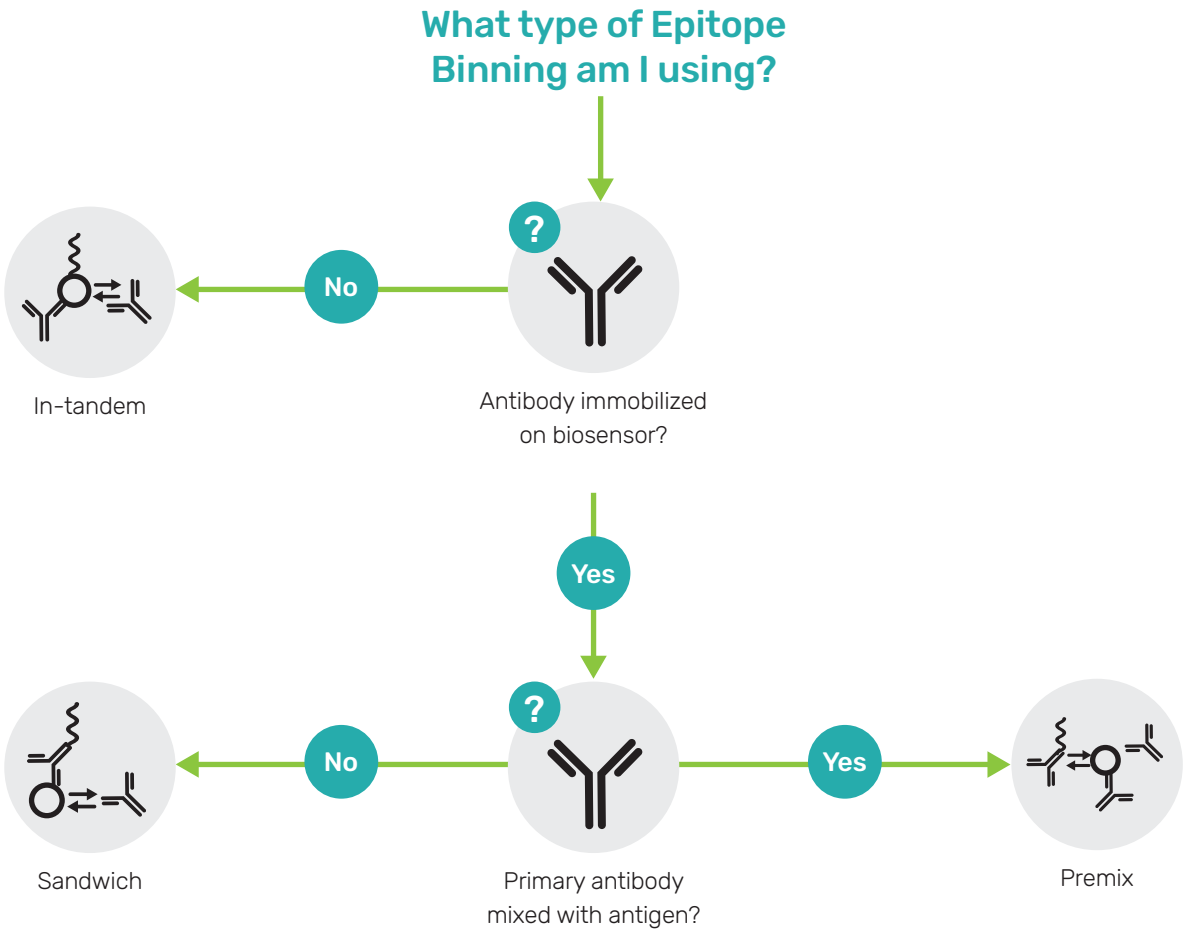


Figure 5: Decision chart for choosing the appropriate epitope binning format.

Part 3: Assay Development

Biosensor selection guideline

In epitope binning studies, choosing the right biosensor is crucial for preserving the structure and activity of the immobilized antigen, captured antibody, or their complexes. The selected biosensor should accurately reflect the binding interactions. Figure 6 offers a decision chart to aid in biosensor selection, while Table 2 lists suitable Gator probes for epitope binning.

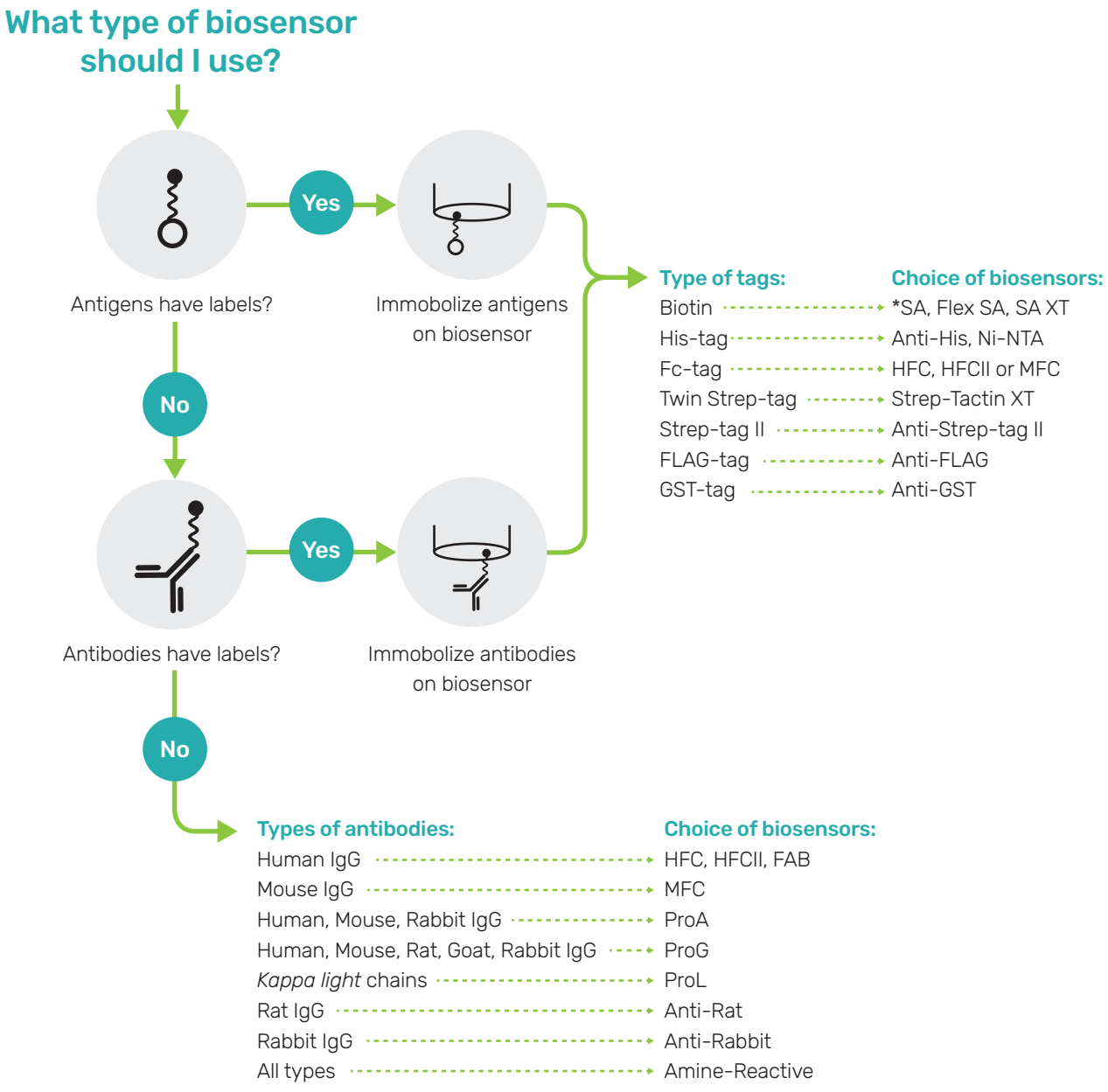


Figure 6: Decision chart for choosing the proper biosensor.
****Choice of Streptavidin-based biosensors is protein dependent.***

Gator® Probes	Function	Applications	Dynamic Range	Regeneration
Protein A (ProA)	Binds IgGs of various species including human and mouse	Q	0.02 - 2000 µg/mL	Yes
Protein G (ProG)	Binds IgGs of various species including human and rat	Q	0.02 - 2000 µg/mL	Yes
Protein L (ProL)	Binds IgGs of various species through the kappa light chain	Q	0.02 - 2000 µg/mL	Yes
Anti-Human IgG Fc (HFC)	Immobilize human Fc-fusion protein or human IgG for quantitative or kinetic analysis	Q/K/QKR/EP	0.05 - 300 µg/mL	Yes
Anti-Human IgG Fc Gen II (HFCII)	Immobilize human Fc-fusion protein or human IgG for quantitative or kinetic analysis	Q/K/QKR/EP	0.3 - 6000 µg/mL	Yes
Anti-Mouse IgG Fc (MFC)	Immobilize mouse Fc-fusion protein or mouse IgG for quantitative or kinetic analysis	Q/K/QKR/EP	0.02 - 2000 µg/mL	Yes
Anti-Human FAB (FAB)	Binds F(ab), F(ab')2, Fc receptor, and full-length Human IgG	Q/K/QKR/EP	0.3 - 3000 µg/mL	Yes
Anti-His (HIS)	Binds His-tagged proteins	Q/K/QKR/EP	Protein-dependent	Yes
Ni-NTA	Tris-NTA and charged with Ni^{2+} ions binding to His-tagged proteins	Q/K/QKR/EP	Protein-dependent typically, 0.25 – 1000 µg/mL	Yes
SA XT	Biotinylated proteins and large molecules	K	Protein-dependent	No
Strep-Tactin XT	Proteins with Twin-Strep-tag®	Q/K	Protein-dependent	No
Streptavidin (SA)	Binds biotinylated and Avi-tagged biomolecules	K/EP	Protein-dependent	No
Flex SA	Binds biotinylated and Avi-tagged biomolecules with reactivable sensor surface	K	Protein-dependent	Reactivable
Amine-Reactive (AR)	Covalently attach amine group of proteins using EDC/NHS	K/EP	Protein-dependent	No
Custom-Made	Custom-made biosensors for your specific applications (Anti-Rat, Anti-Rabbit, Anti-GST, Anti-FLAG and Anti-Strep-tag II)	Varies	Varies	Varies

Table 2: List of Gator probes for epitope binning assays.

Streptavidin-Based Biosensors

Streptavidin-based capture approach is applicable to all epitope binning methods. Gator Bio provides various ready-to-use streptavidin-based biosensors, including SA, SA XT, and FLEX SA. In many cases, commercially available or in-house antigens are already biotinylated, making them suitable for this approach. If the antigen of interest is not biotinylated, a simple biotinylation step can be performed.

However, there are instances when the capture-based approach cannot be used, particularly when working with non-human, non-murine, and untagged antibodies. This situation often arises when dealing with antibodies derived from hamster, rabbit, goat, and sheep. In such cases, the antibodies can be biotinylated and immobilized using streptavidin biosensors. The streptavidin-biotin interaction is highly stable, and these biosensors can be regenerated to the level of the immobilized monoclonal antibody.

In summary, the streptavidin-based capture approach is versatile and widely applicable in epitope binning methods. Gator Bio offers a range of streptavidin-based biosensors for convenient use. While the capture-based approach may not be suitable for certain non-human, non-murine, and untagged antibodies, biotinylation and streptavidin biosensors can still be employed to facilitate epitope binning in these cases.

Amine-Reactive Biosensor

Amine-Reactive biosensor uses EDC / NHS amine coupling chemistry to covalently immobilize primary amine-containing antigens or antibodies onto the surface through stable amide bonding. This method is compatible with most purified monoclonal antibodies (mAbs) but not with antibody supernatants. Though mostly recommended for the premix approach in epitope binning studies, especially when using the BLI-based method and encountering situations where in-tandem and sandwich assays do not show binding, covalent linking of mAbs to the biosensor may distort the antibody structure and reduce activity. Antibody-coupled Amine-Reactive biosensors are typically regenerable to the level of the immobilized mAbs, while antigen-coupled biosensors can also be regenerable in some cases.

Anti-His Biosensor

The polyhistidine protein tag (His-tag), commonly engineered into proteins during cloning, can be used to facilitate recombinant protein purification and detection by exploiting the chelating properties of histidine to an immobilized metal. The Gator Anti-His and Ni-NTA biosensors both can be used to capture His-tag antigens, proteins, antibodies, etc. During the epitope binning assay, especially the in-tandem format these two biosensors are extensively used as they have no non-specific binding to antibodies and can be regenerated multiple times. The in-tandem format also has a shorter assay time as the quench step is not needed in this format.

Anti-Fc Biosensor

Gator Bio has choices of Anti-Fc biosensors in the form of MFC, HFC & HFCII. Some custom probes also available are Anti-Rabbit, Anti-Rat, etc. The Fc tagged biosensors bind to the Fc region of the antibodies specifically through the CH2-CH2 binding region. This binding orientation leaves the antigen binding sites available for binding. The biosensors also show no or low cross reactivity to other species of IgG and bind to all the subtypes. Biosensors like MFC and HFC are best used in the sandwich format. Here the antibodies are captured first followed by a quench step that blocks almost all the remaining IgG binding sites. This format of epitope binning is very useful when the antigen has no tags like His, Biotin, or FLAG, etc.

Assay Design and Optimization

Epitope binning assays are designed based on available sample types. After selecting the appropriate biosensor, a pilot study is recommended to determine optimal concentrations for the antigen, primary antibody, and competing antibodies. Figure 7 provides a decision chart to facilitate this parameter selection process.

In-Tandem Format

This approach is the preferred method for epitope binning in most cases. It utilizes tag-based biosensors to capture antigens, ensuring that most epitopes remain accessible for antibody binding by strategically engineering the tags at specific locations on the antigen. The antigen-biosensor interaction is highly stable, providing a reliable surface for subsequent binding steps. Recommended biosensors for this approach include Anti-His (HIS), Streptavidin (SA), SA XT, Ni-NTA, Anti-Human IgG Fc (HFC), and Anti-Mouse IgG Fc (MFC) probes. Additionally, Gator Bio offers custom biosensors with ready-to-use Anti-FLAG and Anti-GST probes, which can be provided upon request.

To optimize antigen loading on biosensors, aim for a loading height of approximately 0.5 nm-1 nm and extend the loading time to ensure uniform capture reagent distribution. During the initial association step, observe saturation by using a higher concentration of the primary antibody (starting at 10 µg/mL) compared to the competing antibody. Saturation is indicated when the binding curves flatten out or reach a plateau. Subsequently, use one-third of the primary antibody concentration as the starting concentration for competing antibodies. Allow sufficient time to observe some curvature in the data traces, typically around a 2-5 minute association, as reaching equilibrium is not necessary.

Tips for in-tandem format:

1. Load antigens with or without tags (His, Biotin, Strep, FLAG, GST, etc.). If using an untagged antigen directly on an Amine-Reactive biosensor, verify its activity.
2. Ensure primary antibodies fully saturate the biosensor surface and stay bound to antigens to avoid "free antigens" binding to competing antibodies. To prevent ambiguous data, use primary antibodies with strong affinity for the antigen.

Sandwich Format

This format involves capturing the primary antibody onto the biosensor surface. HFC or MFC probes are recommended for their pre-immobilized high-affinity capture antibodies that bind to monoclonal antibodies via the Fc-region, ensuring favorable antibody orientation. For non-human and non-murine antibodies, Protein A, G, and L biosensors are suitable, especially for rabbit antibodies and nanobody capture.

During primary antibody immobilization, achieving saturation is essential, which is observed when binding curves flatten out or reach a plateau. Thoroughly blocking the biosensor surface after immobilization is crucial to prevent false positive outcomes caused by direct binding with competing antibodies. This involves an additional blocking step with 50 µg/mL of irrelevant IgG of the same isotype (if applicable) for 3-10 minutes, followed by a 2-4 minute wash or multiple wash steps to remove loosely-bound antibodies before antigen association. To confirm the absence of additional self-binding, dip the biosensors back into the primary antibody wells and check for binding.

For antigen association, a good starting concentration is 10 µg/mL, ensuring this step also reaches saturation, typically taking about 2-7 minutes. Finally, use one-third of the primary antibody concentration as the starting concentration for the competing or sandwiching antibodies. Allow sufficient time to observe some curvature in the data traces, typically around a 2–5 minute association, as reaching equilibrium is not necessary.

Tips for sandwich format:

1. When using HFC or MFC probes to immobilize primary antibodies, make sure to block the unsaturated biosensor surface with generic Human or Mouse IgG.
2. Be cautious of using primary antibodies with weak affinity for the antigen, as it may result in ambiguous data.
3. If primary antibodies are directly immobilized onto Amine-Reactive or Streptavidin biosensors, ensure that the antibodies still show binding to the antigen.

Premix Format

This format is similar to the sandwich format, but it involves capturing the competing antibody instead of the primary antibody onto the biosensor surface. HFC or MFC probes are recommended for their pre-immobilized high-affinity capture antibodies that bind to monoclonal antibodies via the Fc-region, ensuring favorable antibody orientation. For non-human and non-murine antibodies, Protein A, G, and L biosensors are suitable, especially for rabbit antibodies and nanobody capture.

During competing antibody immobilization, achieving saturation is essential, observed when binding curves flatten out or reach a plateau. Thoroughly block the biosensor surface after immobilization to prevent false positive outcomes caused by direct binding with primary antibodies. Also crucial is the additional blocking step with 50 µg/mL of irrelevant IgG of the same isotype (if applicable) for 3-10 minutes, followed by a 2-4 minute wash to remove loosely-bound antibodies before antigen association. To confirm the absence of additional self-binding, dip the biosensors back into the primary antibody wells and check for binding.

Next, incubate antigens with a concentration at or greater than the dissociation constant (K_D) with a large molar excess of primary antibody to create a premix sample. Introduce this premix sample to the biosensor previously loaded with the competing antibodies. Allow sufficient time to observe some curvature in the data traces during the premix association, typically around a 2-5 minute association, as reaching equilibrium is not necessary.

Tips for premix format:

1. Use an antigen concentration equal to or greater than the dissociation constant (K_D).
2. Ensure a large molar excess of primary antibodies in the premix sample compared to the antigen.
3. If using HFC or MFC probes to immobilize primary antibodies, block the unsaturated biosensor surface with generic Human or Mouse IgG.
4. When directly immobilizing primary antibodies onto Amine-Reactive or Streptavidin biosensors, confirm that the antibodies still show binding to the antigen.
5. Verify that the antibodies can self-block, meaning there is no binding when primary and competing antibodies are the same.
6. Ensure that the antibodies do not cross-react with each other.

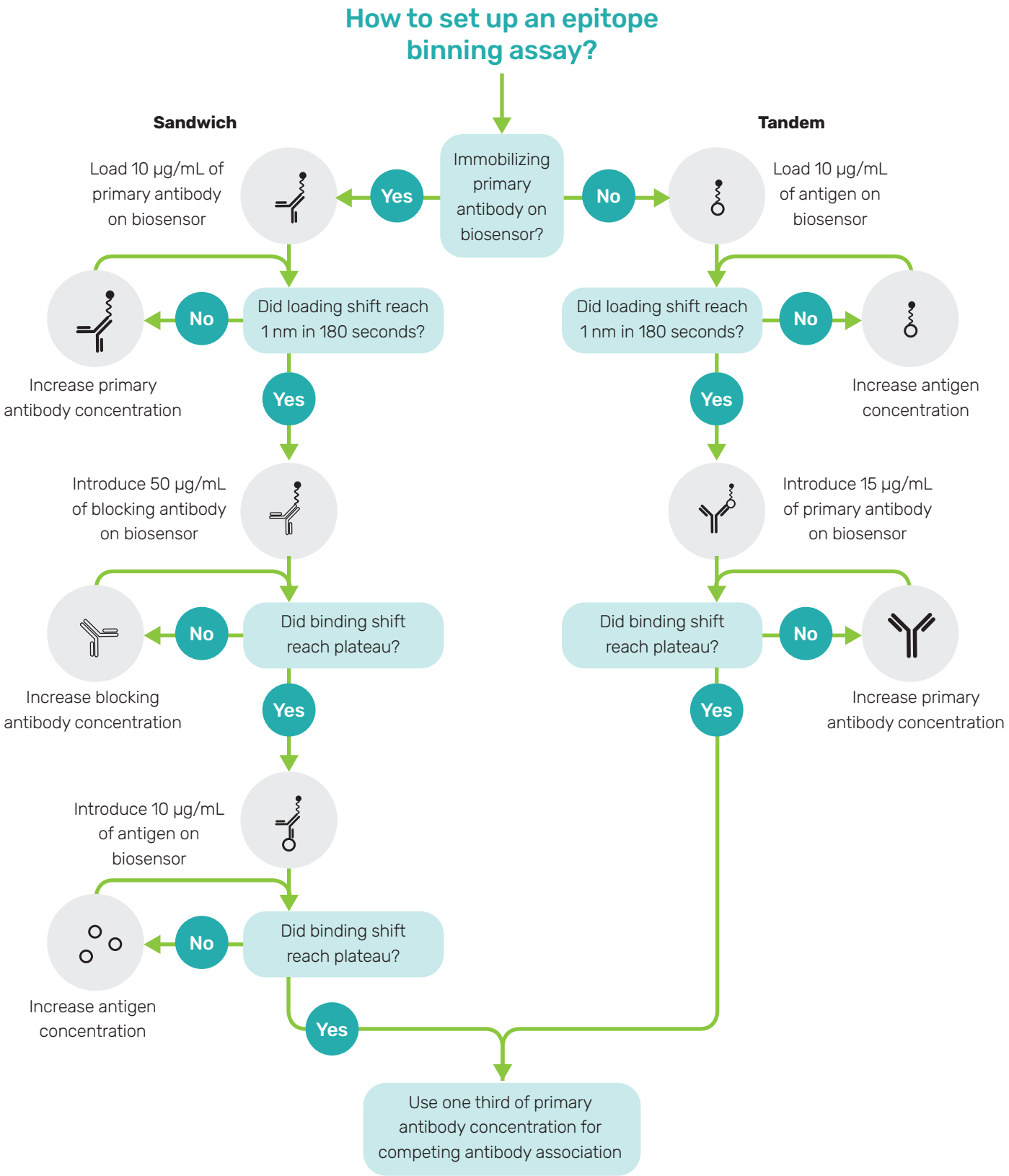


Figure 7: Decision chart for optimizing epitope binning assay.

Part 4: Assay Setup

Epitope Binning Assay Workflow

Typical workflow to set up an assay on the Gator platform involves the following steps:

1. **Sample preparation:** prepare the necessary buffers, regeneration solution, antigen, and antibody solutions, calculating approximate quantities required for the entire assay beforehand.
2. **Biosensor preparation:** hydrate the probes in max plates for at least 10 minutes, ensuring the hydration buffer closely matches the assay running buffer.
3. **Assay design:** decide the layout of the epitope binning experiment, considering the specific Gator instrument being used (e.g., Gator® Prime and Gator® Plus with eight biosensors per assay, or Gator® Pro with 8, 16, 24, or 32 biosensors).
4. **Assay setup:** define the plate layout, assay steps, and biosensors in the GatorOne software assay setup.
5. Sample plate preparation: prepare the sample plate(s) based on the defined plate layout, filling corresponding wells with appropriate buffers, antibodies, antigen, and regeneration solution(s).
6. **Placement:** place the biosensors in the max plate and the assay plate(s) onto the plate station(s) in the instrument for 10 minutes, ensuring complete hydration of the biosensors and uniform assay temperature across the assay plate(s).
7. **Data acquisition:** run the assay, following the established assay steps.
8. **Data analysis:** perform data processing and analysis using the GatorOne software to extract meaningful information from the generated assay data.

Epitope Binning Assay Steps

Common steps during the epitope binning process include:

1. **Pre-wet:** biosensors should undergo a pre-wet step for at least 10 minutes. When using supernatant, biosensors must be pre-wet in media or buffer matching the corresponding samples for 10 minutes before the assay. For purified samples, it is recommended to use Q or K buffer (PN: 120010 and 120011 Gator Bio Inc.).

2. **Baseline:** baselines are established using buffer or media matching the assay for loading and the two association steps to reduce non-specific binding and buffer effects. These steps remove any unbound antigen and antibody from the biosensor. If antibodies are in cell culture supernatants, baseline steps preceding the antibody binding should be performed in mock-transfected supernatants, spent media, or low IgG FBS supplemented in growth media. If non-specific binding occurs, optimize the buffer by increasing BSA or detergent concentration.

   Epitope binning assays typically include baseline steps for: (1) before antigen loading in in-tandem format, (2) before primary antibody loading in sandwich format, (3) before premix sample loading in premix format, (4) prior to the association of competing antibodies in in-tandem format, and (5) prior to the association of antigens in sandwich format.

3. **Loading:** the recommended loading height for both antigen and antibody is approximately 0.5 nm-1 nm. Longer loading time ensures equal distribution of the capture reagent on the surface.

   For capture-based biosensors like HFC or MFC probes, ensure the binding curve reaches saturation, and follow it with a blocking step. If all monoclonal antibodies are of the same isotype,

blocking with 50 µg/mL of irrelevant IgG of the same isotype for 3-10 minutes is sufficient. Afterward, perform a 2–4 minute wash or multiple wash steps to remove any loosely-bound antibodies before antigen association. Confirm the absence of additional self-binding by dipping the biosensors back into the same antibody wells.

4. **Association:** ensure the first association step reaches saturation, and for the second association, allow enough time to observe some curvature in the data traces, but reaching equilibrium is not necessary.

   In in-tandem format, the two association steps are: (1) binding of primary antibodies to the antigen-loaded biosensor and (2) binding of competing antibodies to non-overlapping regions of the antigen-loaded biosensor.

   In sandwich format, the two association steps are: (1) binding of antigens to the primary antibody-loaded biosensor and (2) binding of competing antibodies to non-overlapping regions of the antigens.

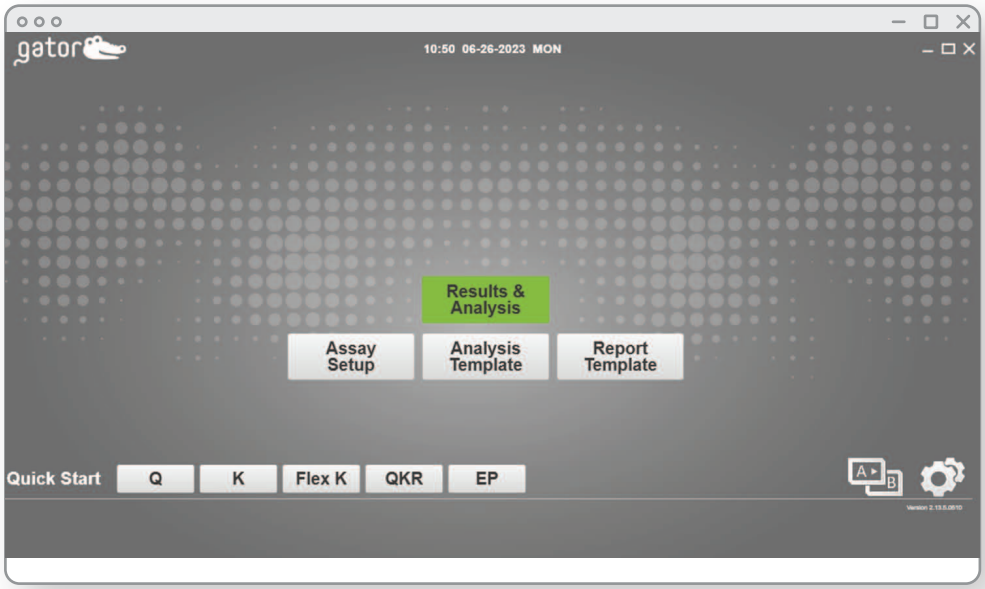
5. **Custom Step:** GatorOne software offers a special custom step that can be inserted between any assay steps. Use this step for additional baselines, surface blocking with reagents, or other necessary procedures.

6. **Regeneration:** Gator Bio's full suite of biosensors offers the advantage of being regenerated and re-used multiple times, leading to significant assay cost savings. Disruption of most antibody-protein interactions can be achieved through a series of short incubations in low pH buffer (pH 1.2–4), such as 10 mM glycine (pH 1.2–3), followed by neutralization in the assay buffer. Specific regeneration conditions for each biosensor are detailed in the product notes. While some biosensors may require further optimization of regeneration conditions, it is important to note that regeneration is not recommended for SA and SA XT sensors. Customized regeneration protocols should be designed based on the specific interacting pairs and the stability of the capture surface.

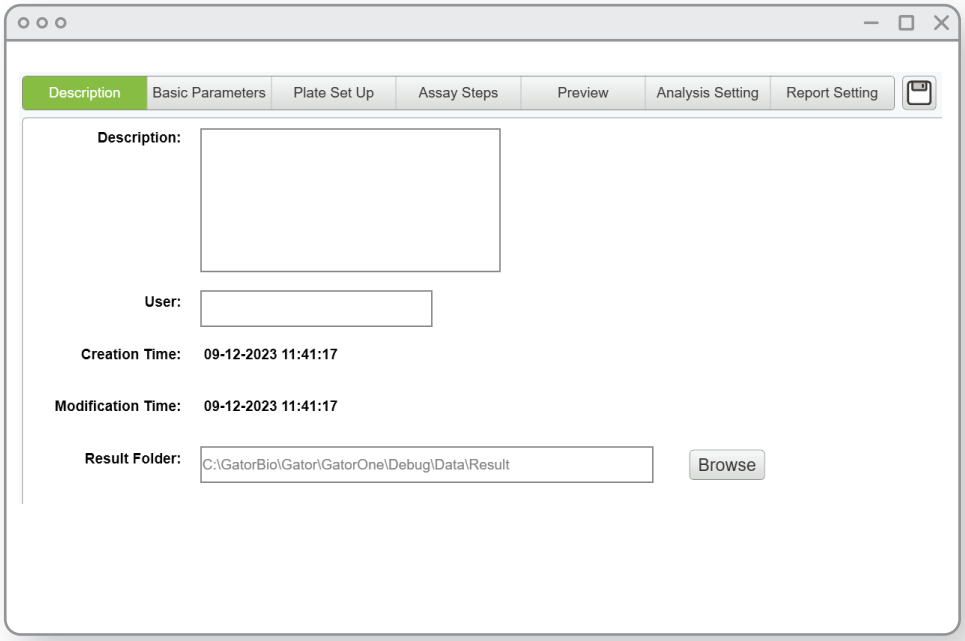
Gator® Prime Assay Setup (8x8 in 96-Well Plate)

Following is an example of an 8x8 epitope binning assay with in-tandem format:

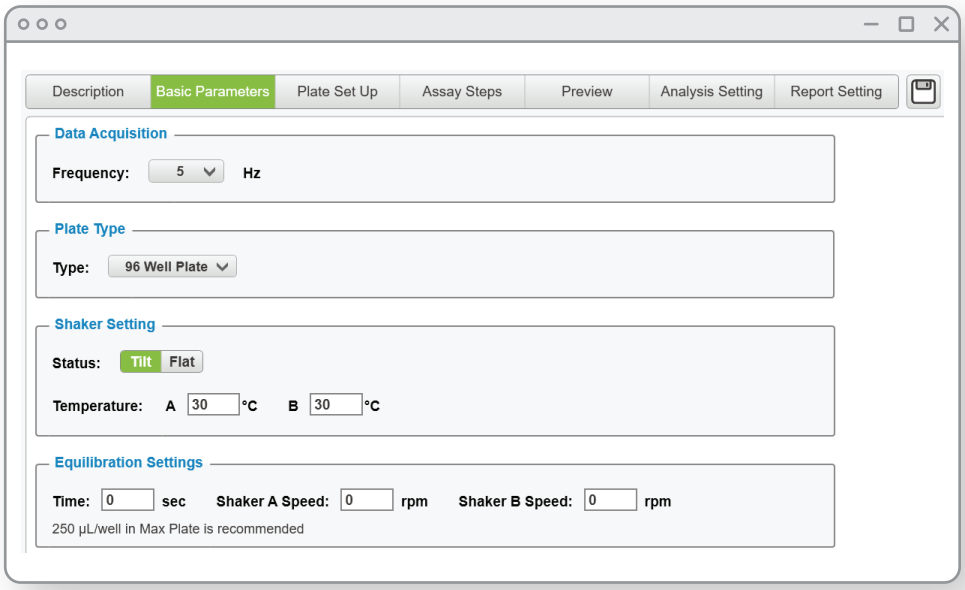
1. Open the assay setup page and click the EP option.



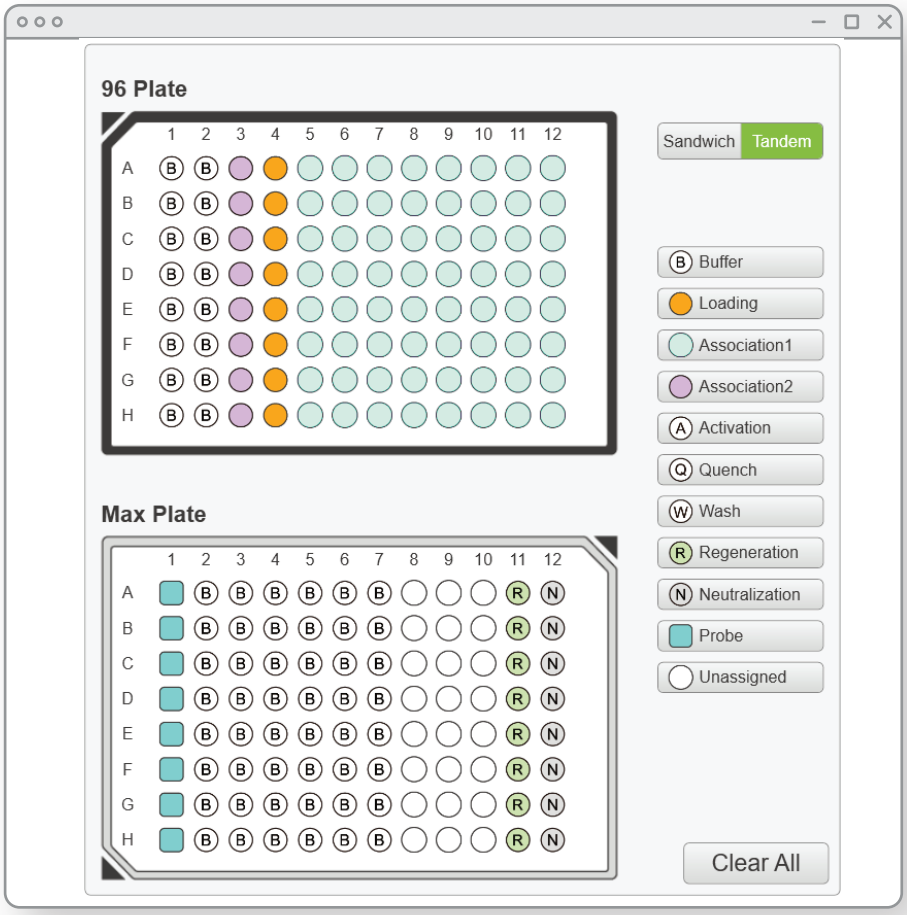
2. Under **Description**, users can keep notes about the assay and set the desired location for results to be saved.



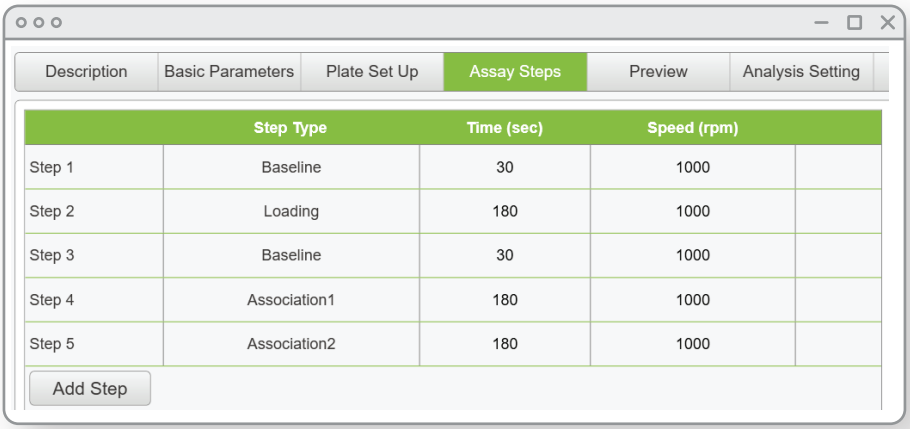
3. Under **Basic Parameters**, users can set up basic parameters, such **Equilibration Settings** to define the time and shaker speed required for pre-wetting the biosensors in the Max plate and pre-conditioning the samples in the Sample plate. Recommended settings for pre-wet are 600 seconds at 1000 RPM for both Shaker A and Shaker Max.



4. Under **Plate Set Up**, users have the flexibility to position the antigens, primary, and competing antibodies as desired. To maximize sample spaces, it is recommended to place wash steps in the max plate. When typing the names of the antibodies, use clear and distinct identifiers such as mAb1, mAb2, etc. This practice facilitates easier analysis in later stages.

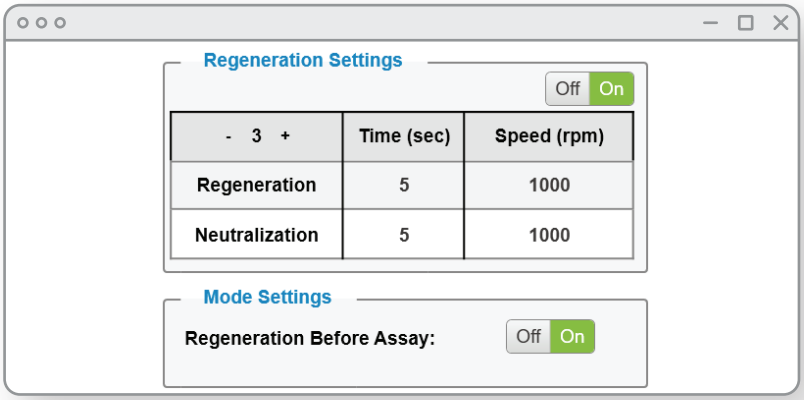


5. Under **Assay Steps**, users can see that the software has a default assay setup. The time and speed can be changed. The **Add Step** option allows users to add custom steps in the assay. Custom steps can also be inserted using the up and down arrow to anywhere in the assay.

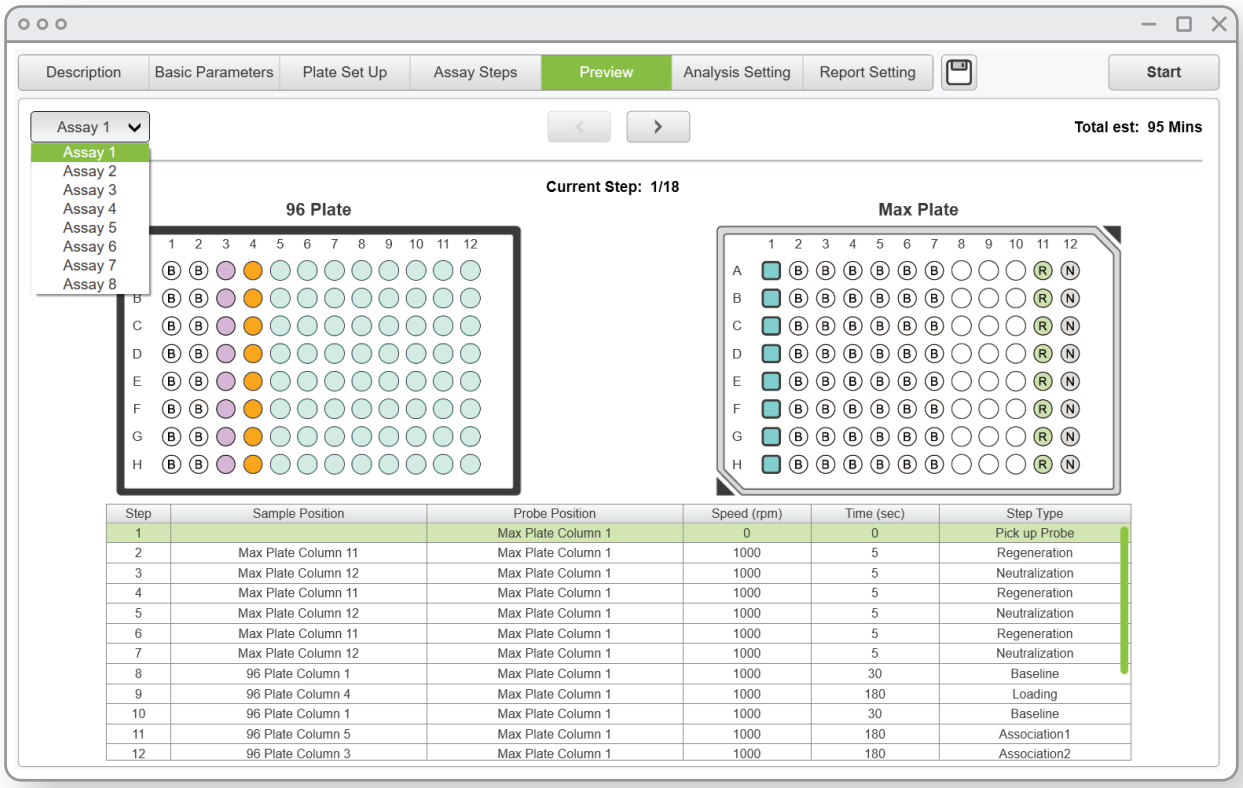


	Step Type	Time (sec)	Speed (rpm)	
Step 1	Baseline	30	1000	
Step 2	Loading	180	1000	
Step 3	Baseline	30	1000	
Step 4	Association1	180	1000	
Step 5	Association2	180	1000	

6. Under the **Regeneration** option, users have the choice to enable or disable regeneration for the desired biosensors. If a biosensor is capable of being regenerated, it is advisable to set up regeneration both before and after assay runs.

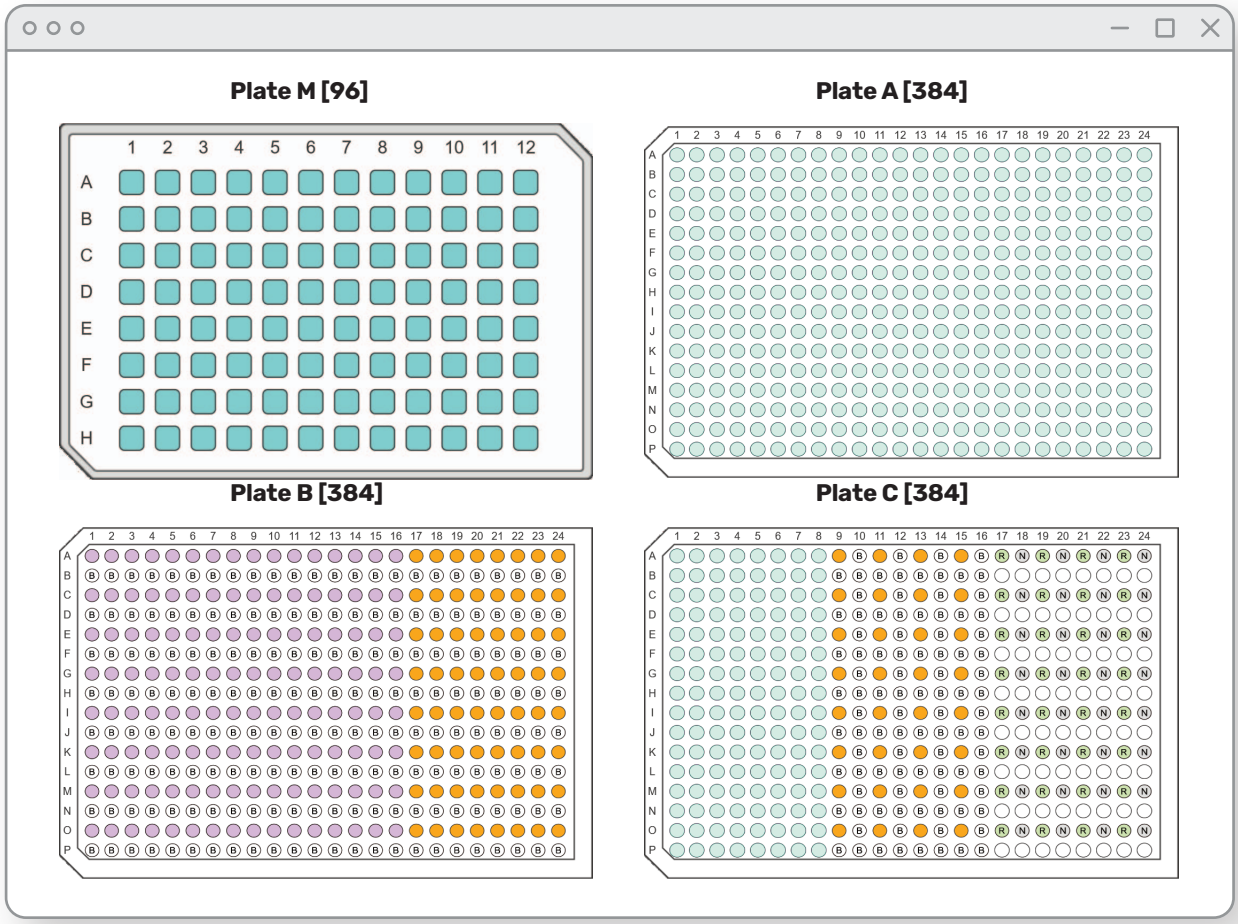


7. Under **Preview**, users can review the assay setup and steps. Before clicking **Start** it is important to confirm that both the Max and Sample plates are properly loaded onto the Gator instrument.



Gator® Pro Assay Setup (32x32 in 384-well Plate):

Following is the plate layout of a 32x32 epitope binning assay in in-tandem format on Gator® Pro Instrument. The assay template files for 8x8, 16x16, 24x24 and 32x32 assays for GatorOne software can be provided upon request.



Sample preparation with Opentrons

Gator Bio has established a strategic partnership with Opentrons, a provider of open-source liquid handling robots. This collaboration aims to offer our users the ability to prepare 384-well sample plates utilizing automated pipetting, enhancing both efficiency and precision. For additional details regarding the integrated Gator-Opentrons protocol file, please refer to Appendix A, where comprehensive information is available to facilitate the optimal use of this automated solution in your experimental setup.

Part 5: Data Analysis

Epitope binning assay data analysis is crucial, but it can be confusing and time-consuming. Traditionally, obtaining meaningful data could take weeks, as it involves determining which antibodies belong to specific bins and understanding their relationships. However, the GatorOne software has streamlined this process, significantly reducing analysis time. Additionally, Gator Bio also offers the NaviGator Software—a software as a service (SaaS) powerful tool for comprehensive epitope binning analysis, enabling researchers to gain deeper insights into the data. Figure 8 presents a useful framework for interpreting epitope binning results.

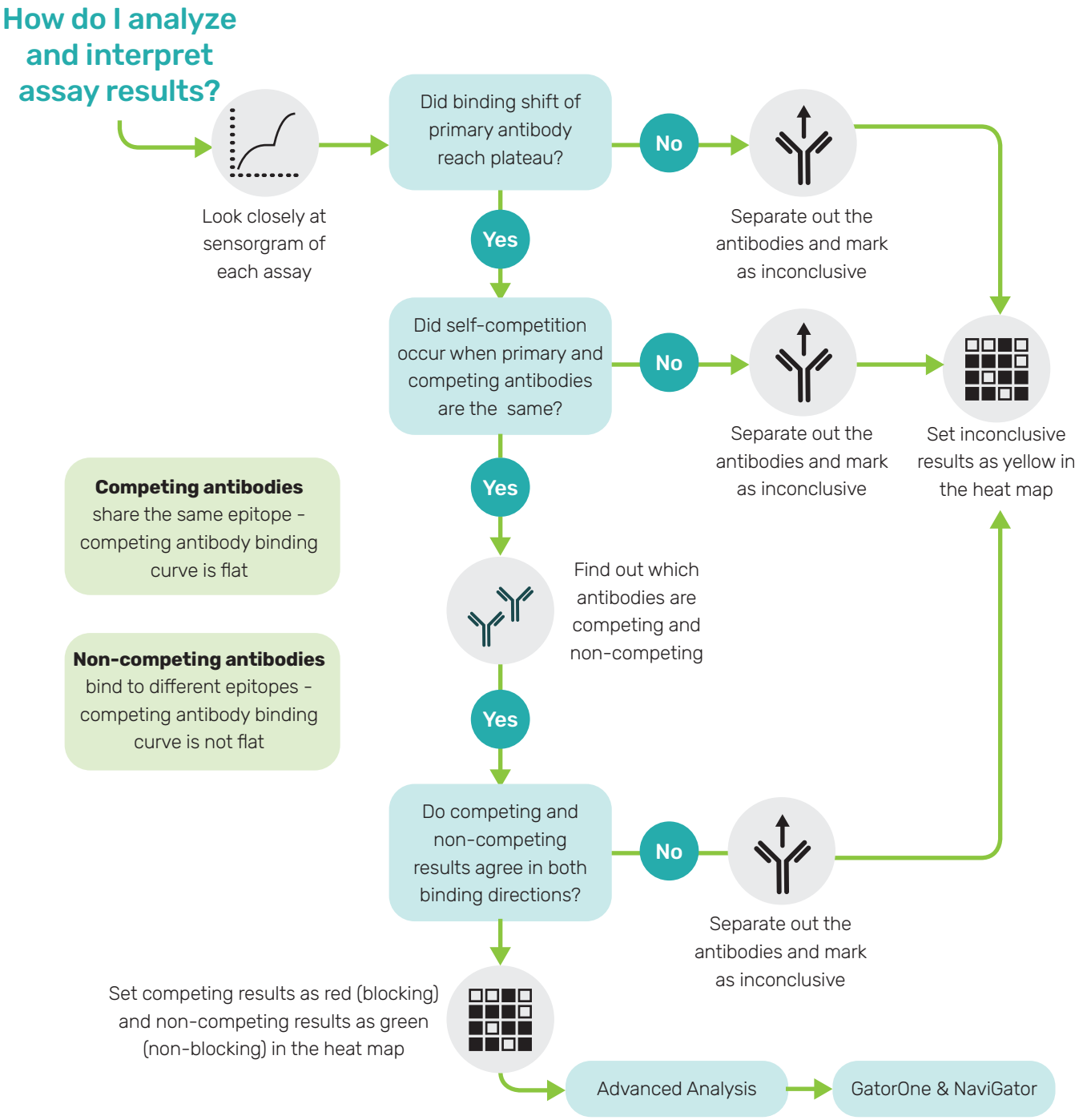


Figure 8: Framework for analyzing epitope binning results.

Looking into the Sensorgrams

After obtaining the epitope binning results, the first thing users should check is the sensorgrams. While a pilot study may have been done to establish assay parameters, it does not guarantee that other antibodies will behave similarly. Unexpected situations may arise, such as lower than expected functional concentration of antibody samples or antibody aggregation. Below are examples of epitope binning sensorgrams and their interpretations:

1. **Saturation curve:** a curve that eventually plateaus or flattens out. This indicates that the biosensor surface has reached saturation with antigens or antibodies, ensuring reliable epitope binning outcomes.
2. **Partial binding curve:** a curve that does not reach a plateau. This may indicate suboptimal antigen or antibody loading on the biosensor surface, resulting in incomplete epitope binning data.
3. **No binding curve:** a flat curve with a slope close to zero, indicating minimal or no interaction between the antigen and antibody. This can be caused by improper immobilization, low affinity, or competition of antibodies.
4. **Aggregation curve:** a curve with a very steep slope and high binding shift that never plateaus.
5. **Self-blocking curve:** a curve showing no binding to themselves suggests that the assay parameters are well-suited and ensures reliable epitope binning outcomes.

Figure 9 illustrates different types of sensorgrams observed based on the presence of blocking and non-blocking antibodies. In Figure 9A, the primary antibody binds to the captured antigen surface, saturating it, while the competing antibodies show no binding, suggesting shared epitopes or non-functionality. Figure 9C depicts the most common scenario with a mixture of blocking and non-blocking antibodies after the primary antibody saturates the antigen surface. Figure 9B arises when the primary antibody exhibits minimal or no binding due to concealed epitopes or non-functionality, possibly from steric hindrance. Understanding these sensorgrams provides valuable insights in epitope binning studies, aiding in characterizing antibody-antigen interactions. It is highly recommended that each assay step should be manually validated for quality.

Figure 10 depicts protein aggregation. A high binding signal is observed but the surface is not saturated. The shift in binding is similar to the binding of a primary antibody but the binding curve never plateaus. Instead, continuous binding is observed suggesting that many bindings sites of the primary antibody are still available. When a competing antibody that shares the same binding site is added, the curve continues to increase even though blocking should be observed.

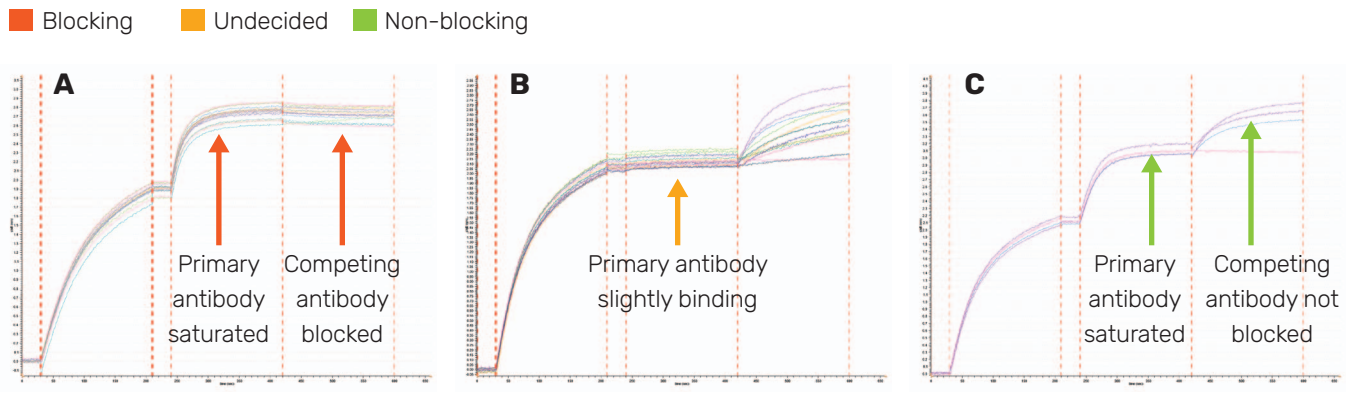


Figure 9. Representative sensorgrams for epitope binning assay. Blocking antibodies (Red – blocking) are shown on the left and recognize identical or very similar epitopes. Non-blocking antibodies (Green – non-blocking) are shown on the right and recognize different epitopes. Inconclusive results (Yellow – undecided) are shown in the middle and can occur when low or no binding is observed for the primary antibody

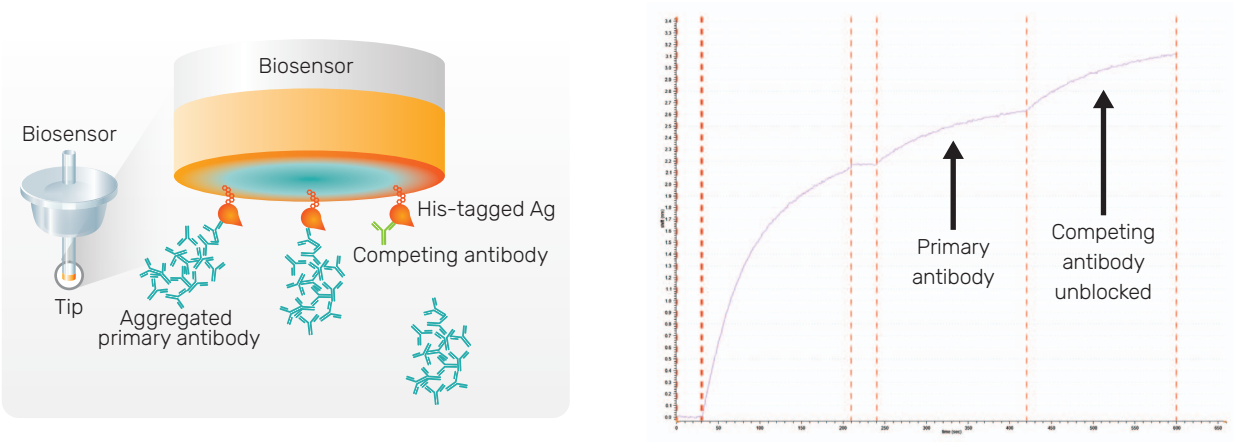
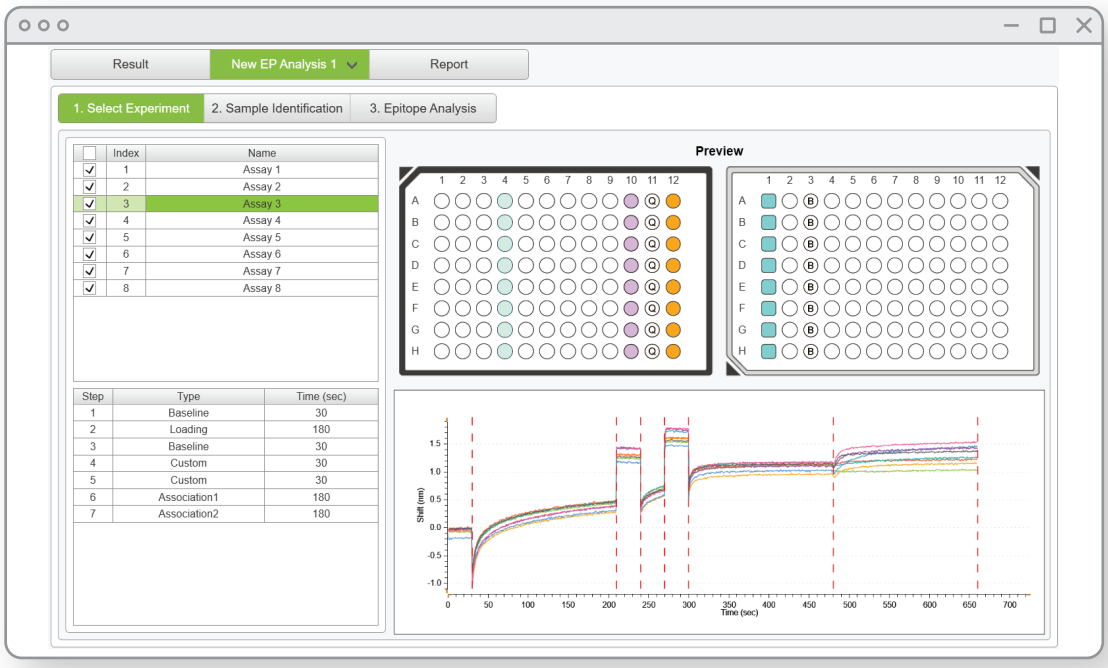


Figure 10: Identifying protein aggregation. Binding occurs with the primary antibody but continues to rise and never plateaus and equilibrates. After the addition of a competing antibody, the binding curve should plateau and show no upward shift. However, a continuous rise indicates protein aggregation is most likely occurring.

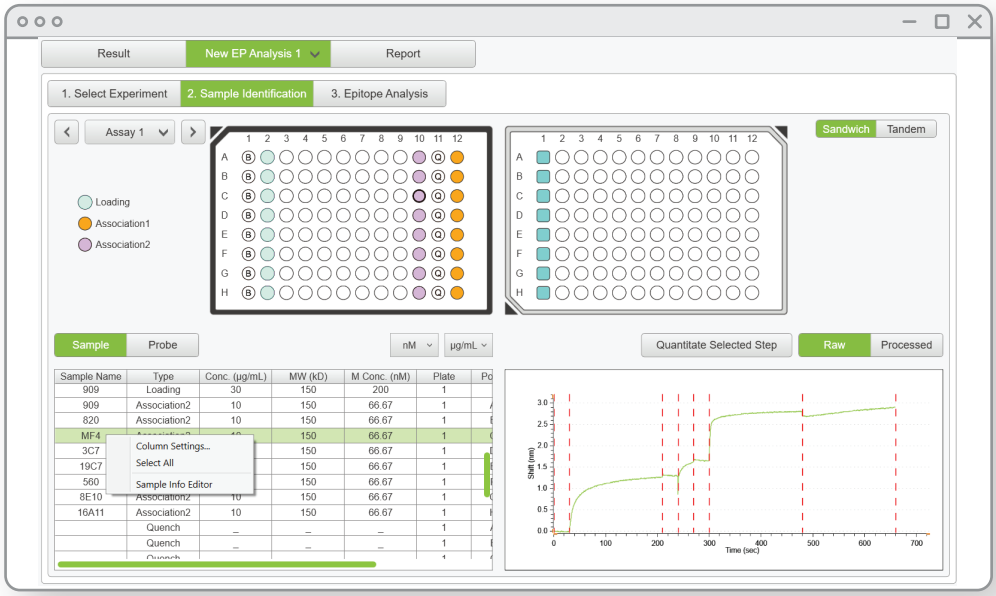
Data analysis on GatorOne

The GatorOne software has easy features to analyze epitope binning data. Below is a step-by-step guide to obtaining a heat map from a sandwich assay:

1. Select the assay(s) that will be evaluated and needed for heat map generation.



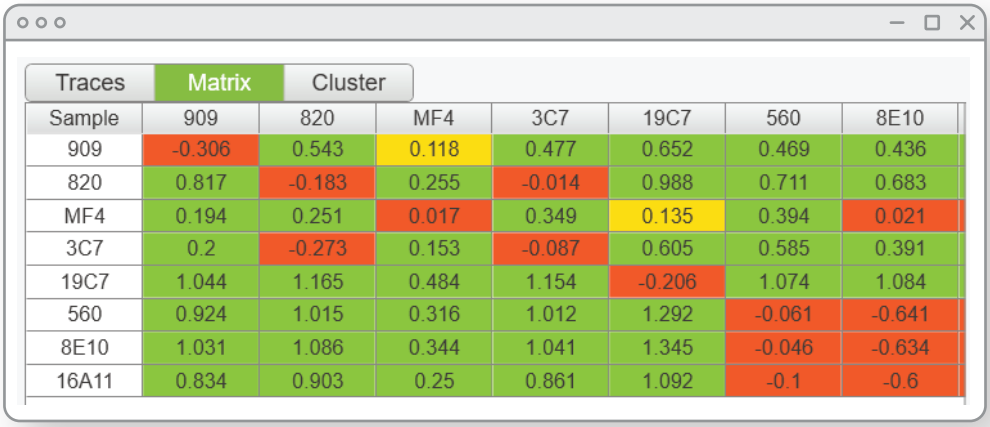
2. Identify the samples by right clicking on the sample column as shown below. It is very important to name the antibodies, otherwise the software cannot provide the heat map.



3. Under **Epitope Analysis**, there are two crucial parameters that need to be set: the **Assoc2 Threshold** and the **Loading Threshold**. The **Loading Threshold** is particularly important because, in the presence of numerous assays, some may show no binding of the capture reagent, which can lead to potentially misleading data. When selecting the **Assoc2 Threshold** value, caution is vital. This value should be based on the primary antibody binding to itself. In most cases, this binding is entirely flat when complete blocking occurs. However, for certain antibodies, residual binding may be observed due to non-specific interactions with the surface.

In this example, the threshold value is set at 0.1 nm. Some antibodies may exhibit ambiguous data because the competition does not provide clear results. In such instances, re-evaluation using an alternative assay format is recommended, as antibodies that compete clearly with the primary antibody likely target different epitopes.

4. Once the values are determined, click on the **classify** button to generate a heat map. In the example provided, the resulting 8x8 matrix clearly depicts the presence of different epitope groups. Antibodies that block each other are grouped together. For instance, in the given example, mAb560 and mAb8E10 share similar epitope groups.



Traces | Matrix | Cluster

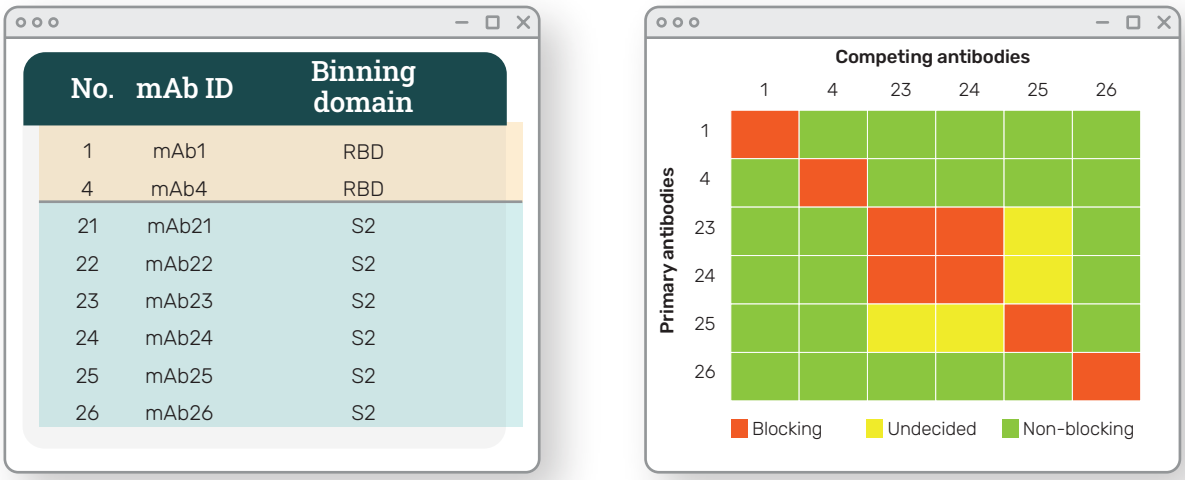
Sample	909	820	MF4	3C7	19C7	560	8E10
909	-0.306	0.543	0.118	0.477	0.652	0.469	0.436
820	0.817	-0.183	0.255	-0.014	0.988	0.711	0.683
MF4	0.194	0.251	0.017	0.349	0.135	0.394	0.021
3C7	0.2	-0.273	0.153	-0.087	0.605	0.585	0.391
19C7	1.044	1.165	0.484	1.154	-0.206	1.074	1.084
560	0.924	1.015	0.316	1.012	1.292	-0.061	-0.641
8E10	1.031	1.086	0.344	1.041	1.345	-0.046	-0.634
16A11	0.834	0.903	0.25	0.861	1.092	-0.1	-0.6

More details about the software analysis can be found in the GatorOne software application note.

What is a heat map?

In the context of epitope binning, a heat map is a graphical representation providing a visual summary of binding patterns between antibodies and antigenic epitopes. It consists of a grid-like structure, with each row representing an antibody and each column an epitope. Colors or shades fill the cells, indicating binding between antibodies and epitopes. The color scale represents binding affinity or interaction levels. For example, a high-intensity color (e.g., dark red) may indicate strong binding, while a low-intensity color (e.g., green) may represent weak or no binding.

Below are examples of heat maps generated by GatorOne software:



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

Figure 11: Heat map from an 8x8 epitope binning assay, where 4 major groups were identified.

By examining the heat map, researchers can identify binding patterns, including shared or distinct epitopes recognized by antibodies. Figure 12 illustrates the logical process of deriving a bin chart from the heat map, allowing for a detailed examination of various interactions.

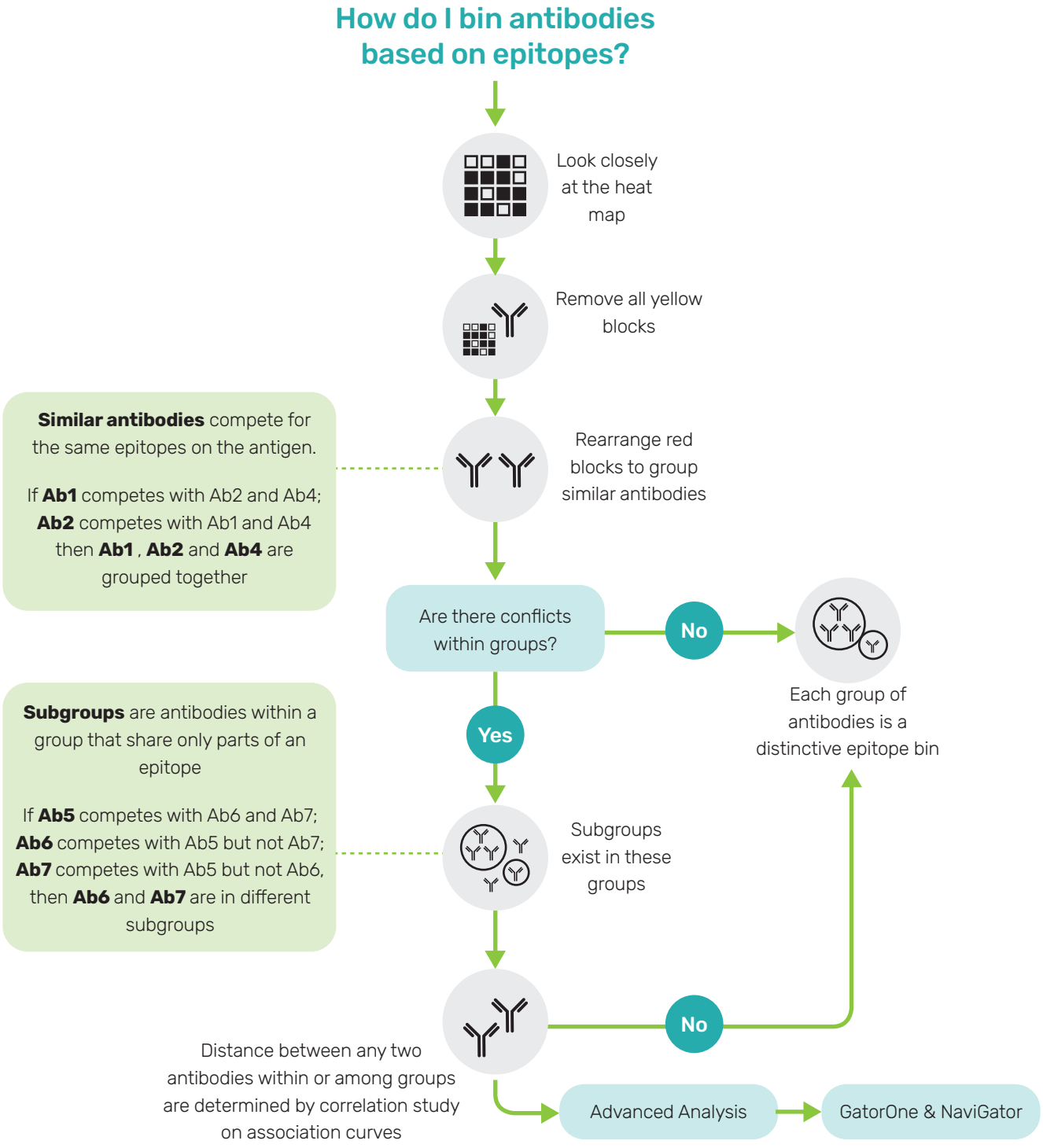


Figure 12. Process of deriving a bin chart from the heat map.

The resulting bin chart provides a clear visualization of different antibodies grouped within distinct epitopes. By analyzing the bin chart, researchers can gain valuable insights into the epitope specificity of antibodies and identify clusters of antibodies targeting similar or overlapping epitopes. This information is crucial for understanding antibody specificity, cross-reactivity, and designing optimal antibody cocktails for diagnostics or therapeutics. Below is an example of a bin chart obtained from a heat map:

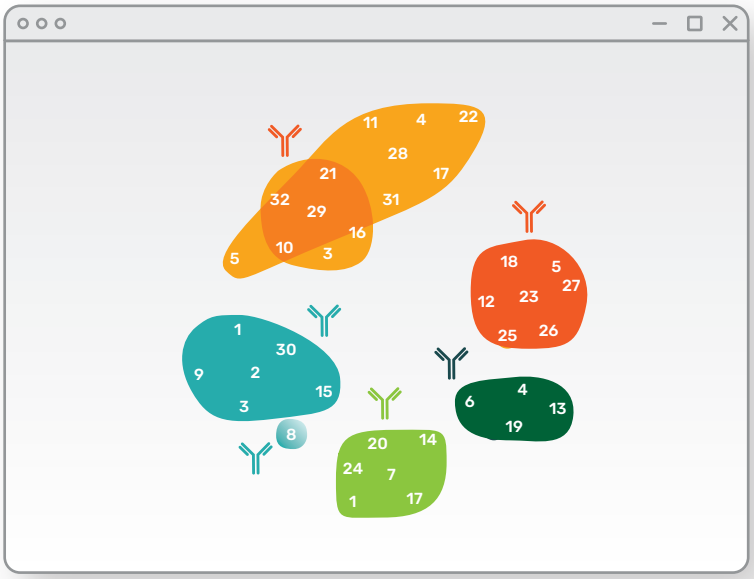


Figure 13. Bin chart from an epitope binning assay, where different bins were identified.

Data analysis on NaviGator

In addition to the GatorOne software, the NaviGator offers SaaS computing and automating complex data workflows. This advanced tool is designed to facilitate a more comprehensive analysis of epitope binning. This software offers users in-depth insights, enabling a richer understanding of antibody-antigen interactions. The NaviGator Software provides an invaluable resource for users seeking to enhance their analytical capabilities in epitope binning studies. For further details and usage guidance, please refer to Appendix B. Below is a heat map generated by the NaviGator Software:

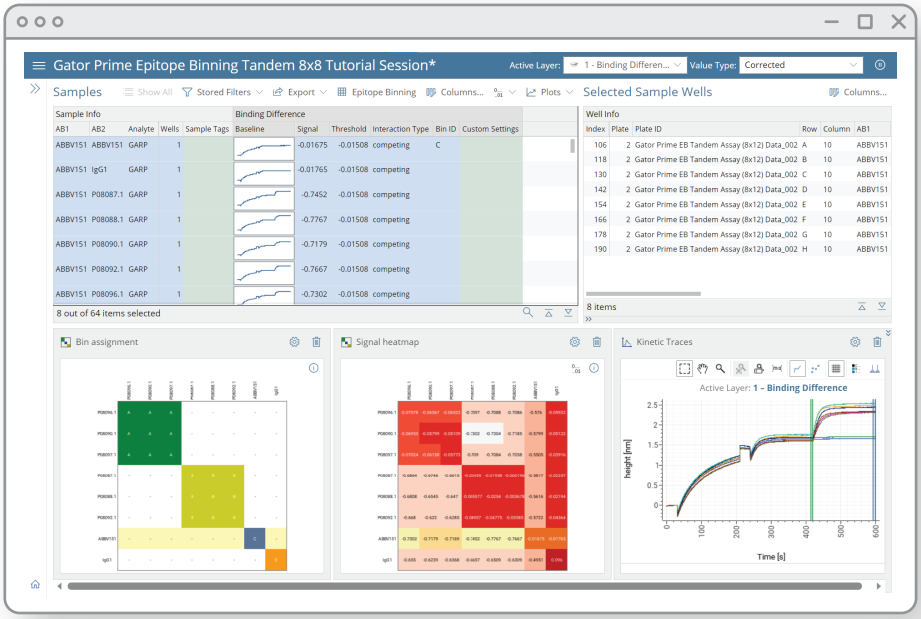


Figure 14: Heat map generated by the NaviGator software.

Part 6: Frequently Asked Questions

General BLI

Question	Answer
What is the difference between SPR, ELISA, and BLI for epitope binning?	They are all methods used in screening monoclonal antibodies. ELISA is mostly used to screen out monoclonal antibodies with lower affinity, specifically to discover antibodies with high affinity. On the other hand, both SPR and BLI are real-time, label-free methods for studying intermolecular reaction kinetics. Antibodies can be detected and ranked based on their k_{off} rates, irrespective of their concentration. Moreover, the BLI method is plate-based, enabling faster and high-throughput screening of antibodies.
Is it safe to use the same column of wells for sample loading, dissociation, regeneration, and neutralization when there are a lot of assays?	It depends on the type of sample you are using. If your samples are crude and may cause non-specific binding, it is best not to reuse the wells.
What buffer should I use, K or Q buffer (or customer buffer)?	If the custom buffer works on the BLI system during the pilot study, it is recommended to continue using it.
Should I always run a control well?	Control wells are always recommended as they provide baseline information for the assay run.

Epitope Binning Assay Design

Question	Answer
Which biosensor should I select?	Refer to **Biosensor Selection Guide** section.
What type of epitope binning assay should I run?	Refer to the decision chart in **Figure 5.**
I have 50 antibodies for epitope binning. Where do I start?	Refer to the flow chart in **Figure 1.**
Do I need to test antibody in both directions?	Yes, to obtain the correct heat map, it is necessary to use the same antibody as the primary and the competing antibody in an assay.
What is a reference antibody?	A reference antibody binds to a known epitope on the target antigen and competes with other antibodies to assess the relative epitope specificity within the antibody panel.
How do I choose a reference antibody?	If you purchased your antibody through a vendor, they should be able to provide information about the reference antibody.
How do I know which method (sandwich or in-tandem) works better for my samples?	It depends on the types of samples available. A pilot study is required for a direct comparison.

Epitope Binning Assay Optimization

Question	Answer
Antigen in-tandem format gives low signal	1. Any signal between 0.2 nm and above is acceptable. 2. Check the antigen signal with other antibodies. 3. If possible, try using a different type of biosensor to capture the antigen. 4. Consider the sandwich assay as another option.
How do I know my binding curves reach saturation?	Refer to **Looking into Sensorgrams section**.
How come my binding curves never reach saturation?	It could be due to the functional concentration of the antibody being lower than expected or the presence of antibody aggregation.
What's the concentration for antigens, primary and competing antibodies? Should we decide the concentration based on the size of the molecules?	Refer to the decision chart in **Figure 7.**
How much time do I need for each step, is there a recommended default setup for the first time running?	Refer to **Epitope Binning Assay Steps** section.
Why should the concentration of primary antibodies be 3 times higher than the concentration of competing antibodies?	The primary antibody concentration is 3 times higher because it needs to be high enough to saturate the biosensor surface. In case the antibody affinity is lower, there are still excess antibodies available for binding.

Data Analysis and Interpretation

Question	Answer
How do I interpret my competition data?	Refer to the framework in **Figure 8.**
How do I know whether it's aggregation?	Refer to **Looking into Sensorgrams** section.
What if the control results have a big range? (For example, a-a is 0.3 nm; but b-b is 1 nm.)	Try a different format and optimize your buffer to rule out non-specific binding. The affinity of the antibodies could also be drastically different.
What if the majority of the results cannot match each other? (For example, a-c has no binding, but c-a shows binding.)	In such cases, the results are inconclusive, and additional assays are required to determine the relationships.
Can I process data in other platforms (such as GraphPad Prism)?	Data can be processed on GatorOne and NaviGator. Refer to the **Data Analysis on NaviGator** section for more information.

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