Biacore™ application guide

Epitope binning in Biacore™ systems

cytiva

Introduction

Therapeutic monoclonal antibodies (mAbs) are a large and growing part of the biopharmaceutical market, accounting for over 70% of biopharmaceutics sales (1). MAbs are used as biotherapeutic antibody drugs, in vaccines, and diagnostic tools. Early-stage antibody drug development efforts often generate many leads, and it is essential to confidently characterize and select candidates for further investigation with appropriate biophysical properties, such as affinity, kinetics and specificity.

Epitope binning is a way of characterizing the binding of mAbs to their target protein, the antigen. In epitope binning, mAbs specific to the same target protein are tested pairwise against all mAbs in a set to assess whether they block one another's binding to a specific site of the antigen or not. The mAbs that block binding to the same epitope are "binned" together.

MAbs within the same bin often function similarly and epitope bins can be used to narrow down the choices investigators to choose from. Although the antibodies bind to the same antigen, they may have different mechanisms of action. This is critical for treating some types of cancers and infectious diseases. For applications, such as designing diagnostic tools or when looking for alternate modes of actions for biotherapeutics, the goal is to find antibodies that bind to different epitopes of the antigen. Epitope diversity is also important to broaden intellectual property protection.

Epitope binning should not be confused with epitope mapping. In the latter antibody binding is tested against separate fragments of the antigen to define the specific epitope on the antigen that an antibody binds to.

The most typical case of epitope binning is a set of monoclonal antibodies directed towards the same antigen. Other or mixed antibody formats can also be subjects to epitope binning, for example Fab fragments vs monoclonals. There are also binning examples of non-antibody binders versus antibodies.

Terminology

Term	Meaning
Capturing molecule	A molecule, usually an antibody, that is covalently coupled to the sensor chip. The purpose of the capturing molecule is to capture the first antibody in sandwich and premix assays, or the antigen in tandem assays.
First antibody	The first antibody to be injected in a pair-wise epitope binning.
Second antibody	The second antibody to be injected in a pair-wise epitope binning. Epitope specificity in a binning experiment is assessed based on the binding level of the second antibody (or antigen + second antibody for premix assays).
Antigen	The protein that the antibodies are specific to.
Blocking	A molecule, usually an antibody, that is used to block free binding sites on the capturing molecule after injection of the first antibody to prevent the second antibody from binding to the capturing molecule.
Sandwich assay	An assay with the antigen bound between two antibodies. The first antibody is coupled to the sensor chip and the second antibody is injected in solution over first antibody and bound antigen.
Premix assay	An assay where the first antibody is coupled to the sensor chip. Second antibody is premixed with antigen and then injected over the first antibody on the sensor chip.
Tandem assay	An assay with antigen coupled to the sensor chip. First and second antibody is then injected in sequence over the antigen.
Unidirectional	When binding of antibody is blocked only in one direction, i.e., when antibody A blocks binding of antibody B, but not the other way around.
Blocking response	Second antibody response when binding is blocked by the first antibody. Ideally, blocking response should be close to zero.
Non-blocking response	Second antibody response when binding is not blocked by the first antibody.
Heat map	A result matrix with the first antibodies on the y-axis and the second antibodies on the x-axis. Displays if the antibodies block each other, or if they bind in the presence of each other.
Bin chart	A figure that illustrates antibody bins as separate segments of a circle. This figure can also illustrate partial blocking and unidirectional binding.

Tips for epitope binning

- Choose an assay format depending on your needs and preferences but primarily based on the properties of the reagents.
- For sandwich and premix assays estimate how much of your first antibody you need to capture/immobilize to obtain high enough responses from the second antibody. This is dependent on the lowest affinity of the antibodies you are examining. For tandem, assessment of antigen immobilization level is needed for the same reasons.
- For efficient blocking and to minimize consumption of blocking reagent, couple low amounts of capturing molecule to the sensor chip.
- Check for non-specific binding from the sample matrix by injecting blank samples (sample matrix without antibody) and assess binding to both the active and reference surfaces.
- A data collection rate of 1 Hz is sufficient. Higher data collection rates increase file sizes without providing additional information.
- Establish that your assay is suitable for its purpose by using a subset of samples and controls before you start extended runs with many samples.
- Before running sandwich and premix assays, test efficiency and drift effect of your blocking reagent.
- If there is drift in your assay, find out what injection is causing the drift. Add a blank cycle to the method using real reagents up to the injection causing the drift and buffer for the rest of the injections. Check if similar drift is obtained for this cycle. If so, it can be used for blank subtraction with potential improvement of the evaluation.

Assay formats

Typically, in an epitope binning experiment, all antibodies are run both as first and second antibody. For example, if four different antibodies are being studied, 16 tests are required to include all combinations, including when first and second antibody is the same (self-blocking).

All three major assay formats — sandwich, premix, and tandem — are supported for epitope binning analysis using Biacore™ systems.

Sandwich assay

In a sandwich assay (Fig 1) the first antibody is coupled to the sensor chip, either covalently or via high affinity capture. The first antibody is followed by an injection of antigen and then an injection of second antibody. Binning results are assessed using the second antibody injection. If the second antibody binds to the antigen which is bound to the first antibody, the sandwich complex is classified as non-blocked, i.e., the antibodies bind to different epitopes and are binned separately. If the second antibody does not bind, the sandwich complex is classified as blocked, i.e., the antibodies bind to the same epitope on the antigen and are thus binned together.

When the assay is run in a capture format, a blocking step is needed to block unoccupied binding sites on the capturing molecule and prevent binding of the second antibody. Binding of second antibody to the capturing molecule will result in false positive response for the second antibody and incorrect conclusions regarding the epitope specificity, i.e., the binding of second antibody would be overestimated. For more information on blocking *see* page 10.

The sandwich assay is a very common format since it is quite generic and consumes relatively low amounts of antibody. The assay development is in most cases the easiest among the three formats.

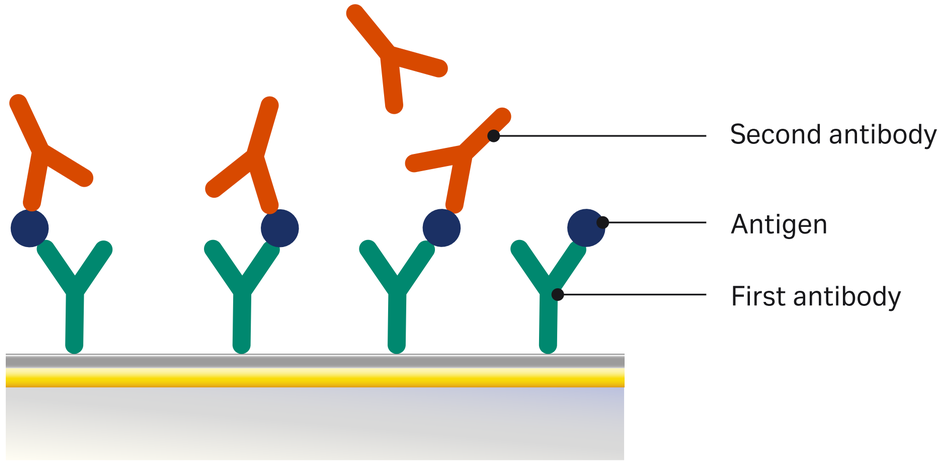

Fig 1. Assay setup sandwich assay.

Premix assay

In a premix assay (Fig 2) the first antibody is attached either covalently or by capture on the sensor chip followed by an injection of a pre-incubated mixture of antigen and second antibody (premix). In the case of capture, a blocking step is added after the first antibody. For this assay format, binning results are assessed using the response from the antigen-second antibody injection. If the antigen second antibody complex binds to the first antibody, the sandwich complex is classified as non-blocked. If the antigen second antibody complex does not bind, the sandwich complex is classified as blocked.

Premix is beneficial when the antigen is multivalent. It also results in higher throughput since it uses fewer injections and each cycle is shorter. The results are usually easy to interpret. To ensure that all antigen binding sites are occupied second antibody should be added in ten times molar excess relative to the antigen.

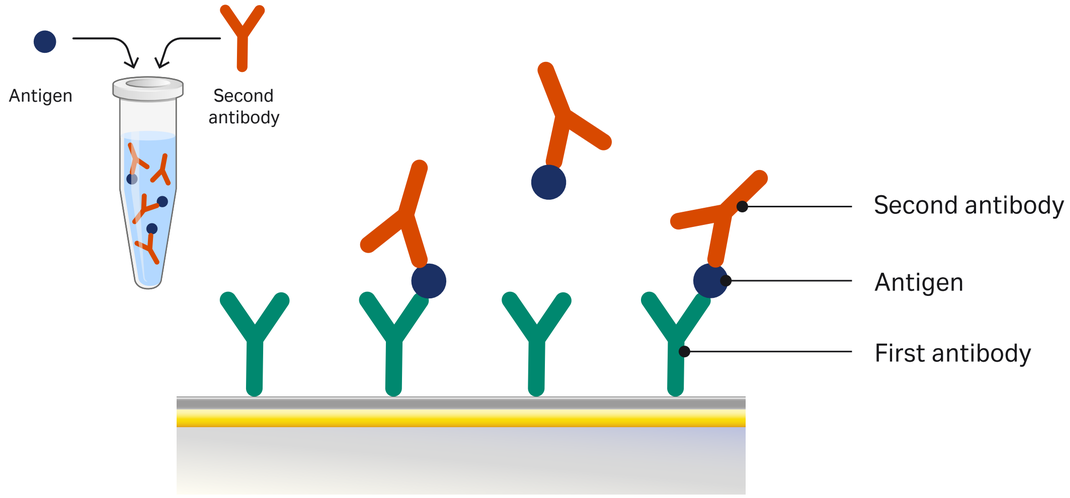

Fig 2. Assay setup premix assay.

Tandem assay

In a tandem assay (Fig 3) the antigen is attached on the sensor chip followed by an injection of first antibody and then an injection of second antibody. Epitope specificity is assessed with respect to the second antibody response. If the second antibody binds to the antigen-first antibody complex, it is classified as non-blocking. If the second antibody does not bind, the complex is classified as blocked.

Tandem is a very straight-forward approach if the interactions have high affinity and the coupling of the antigen does not mask any epitopes involved in the study. If a suitable regeneration is found, tandem assays have the lowest antigen consumption of the three setups.

In a protein there are normally multiple amines present that can couple to an EDC/NHS activated sensor surface. Thus, amine coupling usually results in a more mixed orientation of the antigen on the surface compared to capture. More epitopes are exposed, which is beneficial in an epitope binning situation. For that reason, amine coupling (or another covalent coupling method) is recommended as the primary coupling method in a tandem assay format and not capture.

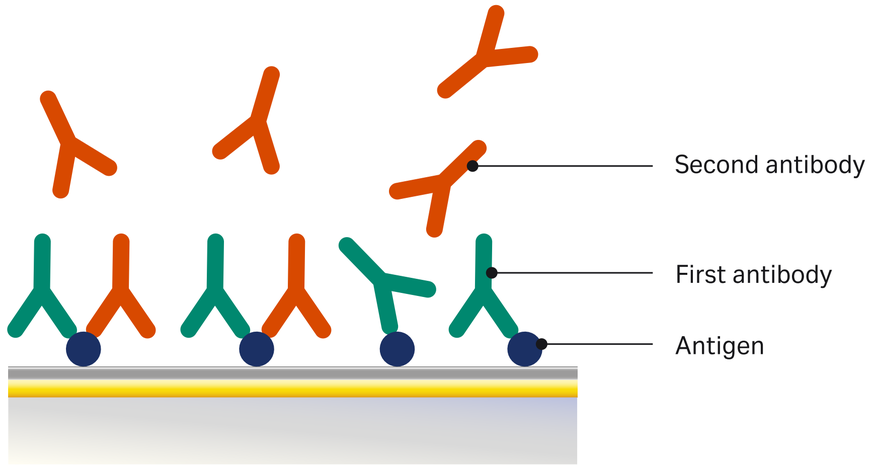

Fig 3. Assay setup tandem assay.

Choice of assay format

Suitable assay formats for your epitope binning experiments will differ depending on nature and available amounts of your reagents. For example, if your antigen is available only in low amounts, the tandem assay with covalently coupled antigen might be the preferred choice over a sandwich assay that requires injection of antigen in every cycle. However, the tandem format requires thorough optimization of regeneration conditions. A sandwich or premix capture format, on the other hand, usually comes with a preoptimized regeneration that omits the need for regeneration scouting. In addition to higher reagent consumption, sandwich and premix assays with capture also require thorough blocking of remaining binding sites on the capturing molecule after capture of the first antibody to prevent the second antibody from binding to the capture surface.

Low affinity of the antigen-first antibody interaction results in dissociation of the antigen and may lead to underestimation of the second antibody binding level. To overcome this problem, antigen and second antibody can be injected using the ***Dual*** command. ***Dual*** injects two solutions in immediate sequence which minimizes time for dissociation of antigen before the second antibody is injected. In cases where the antigen dissociates too rapidly, a premix assay can also be beneficial as there is only one injection involved and no dissociation phase to consider. To ensure that all binding sites on the antigen are completely saturated, a high molar excess of second antibody is required for the premix assay. The molar ratio of second antibody to antigen should be at least 10:1, which means that there is high antibody consumption associated with this assay format.

Choice of assay format should be considered as early as possible during assay development to ensure that the correct parameters are assessed and optimized. Running epitope binning using different assay formats can also be a way of confirming the results.

General considerations

Buffers

HBS-EP+ (HEPES-buffered saline with 3 mM EDTA and 0.05% Surfactant P20) available from Cytiva is recommended as running buffer for epitope binning. If required, samples may be diluted using the same buffer. Precise matching of sample and running buffer is neither needed nor practicable for epitope binning work, since the response read-off is placed after the injection of the second antibody and there are no bulk differences to consider.

Sensor chip preparation

Choice of sensor chip

Sensor chip should be selected with respect to wanted assay format, the presence of any tags on antibodies/antigen and capacity needs.

Sensor surfaces for covalent coupling

The most commonly used sensor chip for covalent coupling of first antibodies or antigens is Sensor Chip CM5. Sensor Chip CM4, Sensor Chip CM3 and Sensor Chip C1 can also be used for coupling of antibodies. Order codes for the respective products are shown in the table (Table 1).

Kits for antibody capture

Capture kits for coupling of mouse and human antibodies and human antibody fragments are available from Cytiva (Table 2). Custom capturing molecules may also be attached if required.

Pre-functionalized sensor chips for capture of antibodies and antibody fragments, such as Sensor Chip Protein A, Sensor Chip Protein L et al., are not recommended for epitope binning. Due to the high capacity of these sensor chips, it is difficult to sufficiently block free binding sites after capture of first antibody to prevent second antibody binding.

Table 1. Sensor chips for covalent coupling of antibodies and antigen

Product	Feature	Product code
Series S Sensor Chip CM5 (pack of 1)	Carboxymethylated dextran surface	29104988
Series S Sensor Chip CM4 (pack of 1)	Carboxymethylated dextran surface. Lower charge	29104989
Series S Sensor Chip CM3 (pack of 3)	Carboxymethylated dextran surface. Shorter dextran chains	BR100536
Sensor Chip C1 (pack of 1)	Carboxymethylated dextran-free sensor chip	29104944

Table 2. Capture kits for antibody capture

Product	Description	Product code
Mouse Antibody Capture Kit	Capture of mouse antibodies	BR100838
Mouse Antibody Capture Kit type 2	Capture of mouse antibodies	29215281
Human Antibody Capture Kit	Capture of human antibodies	BR100839
Human Antibody Capture Kit type 2	Capture of human antibodies	29234600
Human Fab Capture Kit	Capture of human Fab fragments via kappa and lambda light chains	28958325
Human Fab Capture Kit type 2	Capture of human Fab fragments via kappa and lambda light chains	29234601

Sensor chips and kits for antigen capture

Pre-functionalized sensor chips or kits for capture of antigen are available from Cytiva (Table 3). Custom capturing molecules may also be attached if required.

Choice of attachment level for capturing molecules

The attachment level for the capturing molecule should be high enough to generate readily identifiable responses in all subsequent steps of the analysis cycle. To enable sufficient blocking, the level of capturing molecule should not be higher than needed. An attachment level of 300–1000 RU is in most cases high enough to obtain good responses of antibodies and antigen and low enough to facilitate efficient blocking.

Choice of attachment level for first antibody

The attachment level for the first antibody should be high enough to generate readily identifiable responses for the antigen and the second antibody. At the same time, it is desired to keep reagent consumption to a minimum. An attachment level of 300–1000 RU is adequate for most epitope binning experiments.

Choice of attachment level for antigen

The attachment level of antigen should be high enough to generate readily identifiable responses for first and second antibody. For example, an attachment level of 200 RU or lower is adequate for an antigen with a molecular weight of around 30 kDa.

Table 3. Sensor chips and kits for capture of antigen

Product	Description	Product code
Series S Sensor Chip SA	Pre-functionalized sensor chip for capture of biotinylated molecules	29104992
Series S Sensor Chip NTA	Pre-functionalized sensor chip for capture of his-tagged molecules	28994951
Biotin CAPture Kit	Reversible capture of biotinylated molecules	28920234
His Capture Kit	Capture of his-tagged molecules	28995056
GST Capture Kit	Capture of GST-fusion molecules	BR100223

Sample preparation

Samples for epitope binning are typically purified monoclonal antibodies but can also consist of clarified material such as hybridoma culture supernatants or phage display preparations used without further purification.

Depending on antibody affinity and antigen size, drift and disturbances, the required response levels for each injection in the sequence may vary from case to case. Generally, non-blocked binding of second antibody should reach at least 30 RU. It is a task for assay development to set the concentrations and contact times required for each contributing injection in the sequence to reach 30 RU in the final injection.

Antibody concentration

Binding response of the antibodies should be high enough to enable a reliable assessment of the results and to be able to differentiate a blocking from a non-blocking response.

For antibodies with known concentration, a suitable concentration is 100–400 nM. Antibodies that are coupled to the surface can likely be diluted. This parameter should ideally be established during assay development. The concentrations of first and second antibodies do not necessarily need to be the same. For example, in a tandem assay the concentration of the first antibody can often be higher than the concentration of the second antibody to ensure sufficient saturation of the epitope.

For antibodies with unknown concentration, check expression levels by injecting a subset of the antibodies over the surface. If the response levels are too low, try to extend the contact time to bind more antibody to the surface.

Antigen concentration

High antigen concentration is required in epitope binning. The affinity of the antibodies is expected to vary and a high antigen concentration will lead to higher response of the second antibody which facilitates data interpretation.

A suitable starting concentration is 100 nM. If this concentration generates antibody responses that are too low, try higher antigen concentrations, e.g., 200, 500 and 1000 nM. Inject antigen over the surface and test binding with a subset of the antibody clones (~4). Select the lowest antigen concentration that results in a second antibody response level of at least 30 RU.

Capture blocking strategy

The most common approach for first antibody coupling is capture, which enables the first antibody to be varied between cycles. Usually, all antibodies in the study can be captured by the capturing molecule, including the second antibody. This response is not distinguishable from binding to the antigen. Any binding of second antibody to the capturing molecule will be interpreted as false positive binding to the antigen. To prevent binding of the second antibody, free binding sites on the capturing molecule must be blocked with a non-related antibody of the same subclass as the antibodies in the test set. If the test set only contains one subclass, there is no need to block the other subclasses. If antibodies are available in high amounts you can completely saturate the capturing molecule with the first antibody, but this is difficult to accomplish.

Capture blocking is typically done directly after first antibody capture by injecting a high concentration of blocking reagent. The blocking reagent is often a dummy antibody that is known to bind to the capturing molecule with high affinity but does not bind to the antigen. It is very important that all free capture molecules are blocked, otherwise a false positive result will occur for the second antibody. This step should be optimized during assay development.

To test if the blocking injection is sufficient, inject various concentrations of the blocking antibody, e.g., 10, 50, 100 and 200 µg/mL, in sequence with no regeneration between the different concentrations. The lowest concentration that results in a response level of less than 5 RU in subsequent injection of blocking antibody should be selected for the assay.

Use of reference subtraction

From a theoretical point of view, reference subtraction is not needed in epitope binning since the most common read-off is done after injection of second antibody. If the response is read during an injection, which is very rare, a reference subtraction with the response from a flow cell without ligand might be needed to remove buffer-sample bulk response mismatches. Reference subtraction is commonly used to tidy up data and eliminate drift. It can be easier to set cut-offs using reference subtracted data, but the outcome is usually the same both with and without reference subtraction.

In most cases the reference flow cell is prepared by not having the first antibody immobilized. There is normally no need to couple a dummy antibody in the reference flow cell.

Use of blank subtraction

Blank subtraction may be needed if there is significant drift in the sensorgrams after injection of one of the interactants. The magnitude of the drift can vary between different antibodies, which makes it hard to design a working blank subtraction strategy that works for the entire run.

One strategy is to replicate all injections with reagents up until the drift causing injection and then use running buffer in all subsequent injections. If it is not known in advance which injection will cause drift, blank cycles for each reagent injection can be included in the run. During evaluation, a suitable blank cycle is selected and used for subtraction. Representative antibodies included in the study can be used for blank cycles since it can be hard to find good dummy reagents.

Asymmetric epitope binning

Epitope binning is most often performed symmetrically, i.e., all antibodies are tested both as first and second antibodies. Asymmetric binning refers to when the set of first antibodies is different, partially or entirely, from the set of second antibodies. Biacore™ Insight Software is designed to handle this for setup, run, and evaluation. The blocking pattern is regarded as unidirectional. See section Common challenges for more information.

Non-specific binding

To verify that there is no binding of the sample matrix (other than running buffer) to the surface, a control experiment is recommended where only the sample matrix is injected over the sensor surface. If there is non-specific binding from the sample matrix, this can be reduced by diluting the samples with running buffer, typically 1:1. Addition of NSB Reducer (available from Cytiva) at 1 mg/mL can also help to reduce non-specific binding to the dextran matrix.

Common challenges

Multivalent antigens

If the antigen is a homomultimer, tandem and premix are the most suitable assay formats. These assays can be designed so all epitopes on the multimer are saturated by first antibody (tandem) or by second antibody (premix).

For the sandwich format, a second injection of first antibody can be made after the injection of the antigen. This will bind additional first antibody to unoccupied binding sites on the antigen. Binding of second antibody to the same epitope is avoided and thereby a false positive second antibody binding to the antigen.

Fast dissociation

Fast dissociation of antigen from the first antibody in sandwich assays will lower the response from the second antibody because there will be less antigen left on the surface to interact with. This can cause a false negative binding result and be the tipping point between binding and no-binding. The best mitigation is to use ***Dual*** command for the antigen and second antibody injections, which will reduce the time for antigen dissociation. ***Dual*** injects two solutions, the antigen and second antibody in direct sequence which reduces time between the two solutions.

In the tandem assay format, a rapidly dissociating first antibody leaves unoccupied binding sites on the antigen which can result in false positive binding of the second antibody. In this situation the best mitigation is to use ***Dual*** for the first antibody and second antibody injections.

The premix assay format is least sensitive to fast dissociation due to equilibration of antigen and antibody binding before and during injection since only one injection is used. Significant drift due to rapid dissociation may still make the evaluation difficult.

Drift

In epitope binning, downward drift is often caused by dissociation of components in the study. Dissociation of blocking antibody is a typical cause of drift. This is best mitigated by scouting for reagents with stable binding during assay development. Blank cycles that reproduce the drift might also be used to subtract the drifting signal.

Unidirectional blocking

Unidirectional blocking is when results differ depending on which of the two antibodies in a pair is used as first antibody. This frequently occurs in epitope binning and are often caused by experimental deficiencies. One reason could be that there is only data from one direction due to the other sensorgram being excluded for low quality. Another reason may be binning runs where the set of first antibodies is different from the set of second antibodies. There are also situations where steric blocking occurs when one antibody in a pair binds first, but does not occur when the other antibody binds first. This can be because the antibodies bind in different ways or in different, partially overlapping epitopes. Deciding to whether or not to include unidirectional blockers largely depends on the situation and the purpose of the binning experiment. If you are trying to find antibodies that do not bind to the same epitope, such as in a diagnostic kit, it is safer to include unidirectional pairs in the bin definition. When searching for antibodies that bind to the same epitope for therapeutic effect, it is safer to exclude unidirectional pairs from the bin definition.

Displacement

Displacement occurs when the second antibody disrupts the complex of first antibody and antigen instead of adding to it. In sandwich setups this means that when the second antibody binds to the antigen, the first antibody loses its binding to the antigen so the complex of second antibody and antigen dissociates. This results in downward drift during the second antibody injection, which can lead to false blocking results and complicate evaluation.

In the tandem setup, binding of the second antibody can cause faster dissociation of the first antibody. This will have similar effects on the evaluation as displacement in the sandwich setup.

Workflow and settings

Sandwich assay

General steps

The general approach to run epitope binning in the sandwich format is summarized below.

1. Dock a sensor chip and couple first antibody on the active surface using standard coupling procedures. Couple different first antibodies on each active surface if you are using multiple flow cells/channels. Run at least 3 startup cycles using buffer and regeneration to equilibrate the system.
2. Inject antigen over both active and reference surfaces.
3. Inject sample containing second antibody over both active and reference surfaces.
4. Regenerate.
5. Evaluate the results.

Settings

Run settings for epitope binning using sandwich are listed below (Table 4). Use the predefined method if one is provided with your Biacore™ system.

Table 4. Run settings sandwich assay

Parameter	Recommended value	Comments
Antigen injection (Analyte)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Injection type	Low sample consumption	
Contact time	60 to 180 s	Long enough to give confidently measurable response levels without compromising throughput.
Pooling	Recommended	If the antigen can be pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
Capture injection (antibody)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Injection type	Low sample consumption	
Contact time	60 to 180 s	Long enough to give confidently measurable response levels without compromising throughput.
Dissociation time	30 s	Long enough to securely provide a range for read-off.
Pooling	Recommended	If the antibodies can be pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
Regeneration		
Flow rate	20–30 µL/min	To be defined during assay development
Contact time	30–180 s	To be defined during assay development

For assays using ***Dual***, replace separate injections of antigen and second antibody with the ***Dual*** command according to the table (Table 5).

Table 5. Run settings sandwich assay using ***Dual***

Parameter	Recommended value	Comments
Antigen injection (*Dual* A)		
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput.
Pooling	Recommended	If the antigen can be pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
Second antibody injection (*Dual* B)		
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput. Allowed contact time can vary with Biacore™ system model.
Pooling	Recommended	If the antibodies can be pooled, it will conserve position usage and reduce reagent consumption.
Common settings (*Dual* A and B)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Dissociation time	30 s	Long enough to securely provide a range for read-off.
Molecular weight	Not required	
Concentration	Not required	
Regeneration		
Flow rate	20–30 µL/min	To be defined during assay development
Contact time	30–180 s	To be defined during assay development

Sandwich assay with capture

The general approach to run epitope binning in a sandwich assay format using capture is summarized below.

1. Dock a sensor chip and attach the capturing molecule on active and reference surfaces using standard coupling procedures. Run at least 3 startup cycles using buffer and regeneration to equilibrate the system.
2. Inject first antibody over the active surfaces. Capture different first antibodies on each active surface.
3. Inject capture blocking agent over the active and reference surfaces.
4. Inject antigen over both active and reference surfaces.
5. Inject sample containing second antibody over both active and reference surfaces.
6. Evaluate the results.

Settings

Run settings for epitope binning using sandwich with capture are listed below (Table 6). Use the predefined method if one is provided with your Biacore™ system.

Table 6. Run settings sandwich assay using capture

Parameter	Recommended value	Comments
First antibody capture (Capture)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Injection type	Capture	
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput.
Pooling	Recommended	If the antibodies can be pooled, it will conserve position usage and reduce reagent waste.
Molecular weight	Not required	
Concentration	Not required	
Blocking injection (General)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Contact time	120 to 240 s	Long enough to give blockage of all remaining capture sites.
Injection type	Low sample consumption	
Pooling	Yes	
Molecular weight	Not required	
Concentration	Not required	
Antigen injection (Analyte)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Injection type	Low sample consumption	
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput.
Pooling	Recommended	If the antigen can be pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
Second antibody injection (Analyte)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Injection type	Low sample consumption	
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput.
Dissociation time	30 s	Long enough to securely provide a range for read-off.
Pooling	Recommended	If the antibodies can be pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
Regeneration		
Flow rate	20–30 µL/min	To be defined during assay development
Contact time	30–180 s	To be defined during assay development

For assays using capture and ***Dual***, replace separate injections of antigen and second antibody with the ***Dual*** command according to Table 5.

Premix assay

The general approach to run epitope binning in a premix format is summarized below.

1. Dock a sensor chip and attach the first antibody on the active surfaces using standard coupling procedures. Couple different antibodies on each active surface. Run at least 3 startup cycles using buffer and regeneration to equilibrate the system.
2. Mix antigen with second antibody and inject over the active and reference surfaces.
3. Regenerate.
4. Evaluate the results.

Settings

Run settings for epitope binning using premix are listed below (Table 7). Use the predefined method if one is provided with your Biacore™ system.

Table 7. Run settings premix assay

Parameter	Recommended value	Comments
Antigen + second antibody injection (Analyte)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Injection type	Low sample consumption	
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput.
Dissociation time	30 s	Long enough to securely provide a range for read-off.
Pooling	Recommended	If the antigen + second antibody can be pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
Regeneration		
Flow rate	20–30 µL/min	To be defined during assay development
Contact time	30–180 s	To be defined during assay development

Premix using capture

The general approach to run epitope binning in a premix format with capture is summarized below.

1. Dock a sensor chip and attach the capturing molecule on active and reference surfaces using standard coupling procedures. Run at least 3 startup cycles using buffer and regeneration to equilibrate the system.
2. Inject first antibody over the active surfaces. Capture different first antibodies on each active surface.
3. Inject capture blocking agent over all surfaces.
4. Inject antigen mixed with second antibody over the active and reference surfaces.
5. Regenerate.
6. Evaluate the results.

Note: mixing of antigen with second antibodies should be done before the run is started to ensure binding is fully equilibrated.

Settings

Run settings for epitope binning using premix and capture are listed below (Table 8). Use the predefined method if one is provided with your Biacore™ system.

Table 8. Run settings premix assay using capture

Parameter	Recommended value	Comments
First antibody capture (Capture)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Injection type	Capture	
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput.
Pooling	Recommended	If the antibodies can be pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
Blocking injection (General)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Contact time	120 to 240 s	Long enough to give blockage of all remaining capture sites.
Injection type	Low sample consumption	
Pooling	Yes	Reagent
Molecular weight	Not required	
Concentration	Not required	
Antigen + second antibody injection (Analyte)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Injection type	Low sample consumption	
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput.
Dissociation time	30 s	Long enough to securely provide a range for read-off.
Pooling	Recommended	If the antigen + second antibody can be pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
Regeneration		
Flow rate	20–30 µL/min	To be defined during assay development
Contact time	30–180 s	To be defined during assay development

Tandem assay

The general approach to run epitope binning in a tandem format is summarized below.

1. Dock a sensor chip and attach the antigen on the active surfaces using standard coupling procedures. Run at least 3 startup cycles using buffer and regeneration to equilibrate the system.
2. Inject first antibody over the active and reference surfaces.
3. Inject second antibody over the active and reference surfaces.
4. Regenerate.
5. Evaluate the results.

Settings

Run settings for epitope binning tandem are listed below (Table 9). Use the predefined method if one is provided with your Biacore™ system.

Table 9. Run settings tandem assay

Parameter	Recommended value	Comments
First antibody injection (Analyte)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Injection type	Low sample consumption	
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput.
Pooling	Recommended	If the antibodies can be pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
Second antibody injection (Analyte)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Injection type	Low sample consumption	
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput.
Dissociation time	30 s	Long enough to securely provide a range for read-off.
Pooling	Recommended	If the antibodies can be pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
Regeneration		
Flow rate	20–30 µL/min	To be defined during assay development
Contact time	30–180 s	To be defined during assay development

For assays using ***Dual*** replace separate injections of antigen and second antibody with the ***Dual*** command according to the table below (Table 10).

Table 10. Run settings tandem assay using ***Dual***

Parameter	Recommended value	Comments
First antibody injection (*Dual* A)		
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput.
Pooling	Recommended	If the antigen can be pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
Second antibody injection (*Dual* B)		
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput. Allowed contact time may vary with Biacore™ system model.
Pooling	Recommended	If the antibodies can be pooled, it will conserve position usage and reduce reagent consumption.
Common settings (*Dual* A and B)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Dissociation time	30 s	Long enough to securely provide a range for read-off.
Molecular weight	Not required	
Concentration	Not required	
Regeneration		
Flow rate	20–30 µL/min	To be defined during assay development
Contact time	30–180 s	To be defined during assay development

Tandem assay using capture

The general approach to run epitope binning in a tandem format using capture is summarized below. Coupling of the antigen to the sensor surface via capture typically results in a uniform orientation of the antigen. Thus, epitopes close to the point of surface attachment risk being inaccessible and unavailable for antibody binding which could generate false negative results. Covalent coupling of the antigen generally results in more mixed orientation coupling of the antigen and less risk of blocked epitopes on all antigen molecules and is preferred over capture for tandem assays. The antigen capture should be checked for stability since drifts make the evaluation difficult.

1. Dock a pre-functionalized sensor chip or attach the capturing molecule on the active surfaces using standard coupling procedures. Run at least 3 startup cycles using buffer and regeneration to equilibrate the system.
2. Inject first antibody over the active and reference surfaces.
3. Inject second antibody over the active and reference surfaces.
4. Regenerate.
5. Evaluate the results.

Settings

Run settings for epitope binning tandem assay using capture are listed below (Table 11).

Table 11. Run settings tandem assay using capture

Parameter	Recommended value	Comments
Antigen injection (Capture)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Injection type	Capture	
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput.
Pooling	Recommended	If the antigen is pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
First antibody injection (Analyte)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Injection type	Low sample consumption	
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput.
Pooling	Recommended	If the antibodies can be pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
Second antibody injection (Analyte)		
Flow rate	10 µL/min	Higher flow rates consume more sample.
Injection type	Low sample consumption	
Contact time	60 to 180 s	Long enough to give measurable response levels without compromising throughput.
Dissociation time	30 s	Long enough to securely provide a range for read-off.
Pooling	Recommended	If the antibodies can be pooled, it will conserve position usage and reduce reagent consumption.
Molecular weight	Not required	
Concentration	Not required	
Regeneration		
Flow rate	20–30 µL/min	To be defined during assay development
Contact time	30–180 s	To be defined during assay development

For assays using capture and ***Dual***, replace separate injections of antigen and second antibody with the ***Dual*** command according to Table 10.

Evaluation of epitope binning

Evaluation tools

The evaluation guidelines described in this application guide follow the workflow in Biacore™ Insight Software and the application specific software extension, Biacore™ Insight Epitope Binning Extension provided by Cytiva. The epitope binning evaluation tool contains four items, an overlay of all sensorgrams zoomed in on the injection used for evaluation, a heat map, a bin chart, and a table. Information on how to work with these items is given in the sections below.

Overview

- Check QC plots and remove disturbed and non-relevant data.
- In the sensorgram overlay (Fig 4), adjust the response read-off range if needed. Most often the default read-off range placed directly after the end of the second antibody injection is suitable.
- Adjust lower and upper cut-offs if needed.
- Inspect data by viewing selections based on for example first antibody. Selection is most efficiently done in the heat map. Remove sensorgrams that disturb the evaluation.
- Include unidirectional blockers in the bin definition if asymmetric data sets are evaluated.
- If uncertain blockers are left, try to decide whether these can be assessed as blockers, non-blockers, or if they should remain uncertain but included in a bin definition.
- Assess the result in the heat map (Fig 5) and the bin chart (Fig 6).

Quality control and selection of data

- Open your run together with a suitable evaluation method for epitope binning.
- If you used a reference surface, start by checking for binding to reference.
- Move on to the epitope binning item and examine the sensorgram overlay. Exclude bad and disturbed sensorgrams and use quality control items if required.
- Confirm that all injection assignments are correct. Sandwich and tandem assays are evaluated based on second antibody responses. Premix assays are evaluated based on antigen-second antibody mix responses.
- Unselect data that should not be included in the evaluation, such as buffer injections not used in the evaluation.

Working with sensorgram overlay

Selection of cut-off level

In the sensorgram overlay (Fig 4) there is an upper and a lower cut-off line. The purpose of these cut-off lines is to separate binding responses from non-binding responses with respect to the second antibody injection, or for the premix assay setup, second antibody mixed with antigen.

Assuming that the assay is correctly designed, sensorgrams with the same antibody as both first and second antibody should have a blocked non-binding response since an antibody should not be expected to bind a second time to the antigen. It is usually practical to place the lower, non-binding cut-off immediately above this group.

Ideally, there should be a gap between binding and non-binding responses which makes placing the upper binding cut-off straight-forward. As shown in the example below, low antigen binding, varying drift and displacement can make this gap disappear which makes an unambiguous placement of the cut-off difficult. Adjusting the read-off range and removing disturbing sensorgrams and interactants may help in some cases. An example will be shown at the end if this application guide.

Normalization

If the antigen binding level varies significantly between different first antibodies when running the sandwich assay format, it may help to normalize the sensorgrams with respect to antigen binding level. Difficulties are often caused by overlapping binding and non-binding sensorgrams when overlaying. Normalization does not change the shape of the sensorgrams; the overlap remains and resolution between binding and non-binding is not improved.

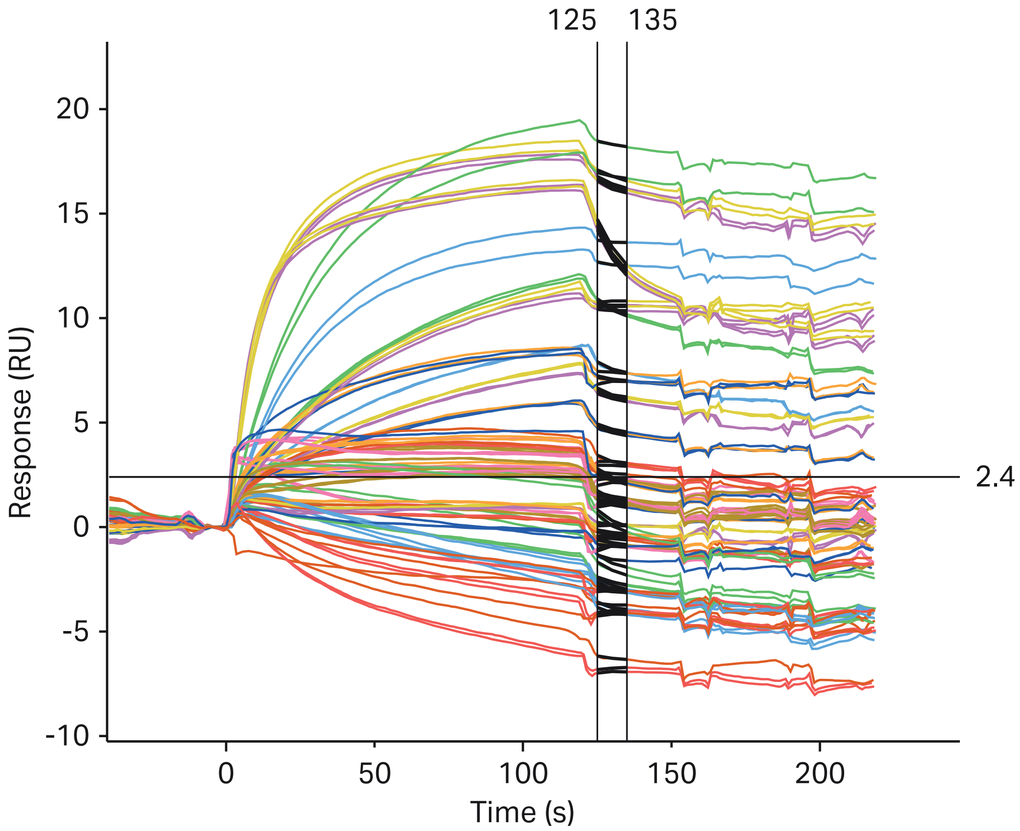

Fig 4. Sensorgram overlay of second antibody injections. Due to low antigen binding and varying drift the gap between non-binding and binding responses is hard to identify which complicates setting of the upper cut-off for identification of binding.

Working with the heat map

A very common way of visualizing epitope binning data is in a heat map (Fig 5). The heat map consists of a matrix of squares in which each square represents a first and second antibody pair. The color (or other expression) of the square indicates status of the interaction. For example, a red square means blocking, i.e., first and second antibody do not bind simultaneously to the antigen. If they do bind simultaneously the interaction is considered non-blocking, and the squares are instead white. There may be other signs and expressions, for example a bold frame around squares where first and second antibody are the same, indicating self-blocking, or an arrowhead indicating that the antibodies in the pair do not give the same result if injected in reverse order, indicating unidirectional blocking. Interactions classified as uncertain are yellow in the heat map.

When evaluating bin data, the heat map can be sorted by run order. Sometimes this can make it difficult to see binding patterns. The default option is to sort by bin. The bins can be identified as the areas along the diagonal that are red (blocking). An example of this is shown in the figure below. The identities of the antibodies in the bins can be read from the y- and x-axes of the map.

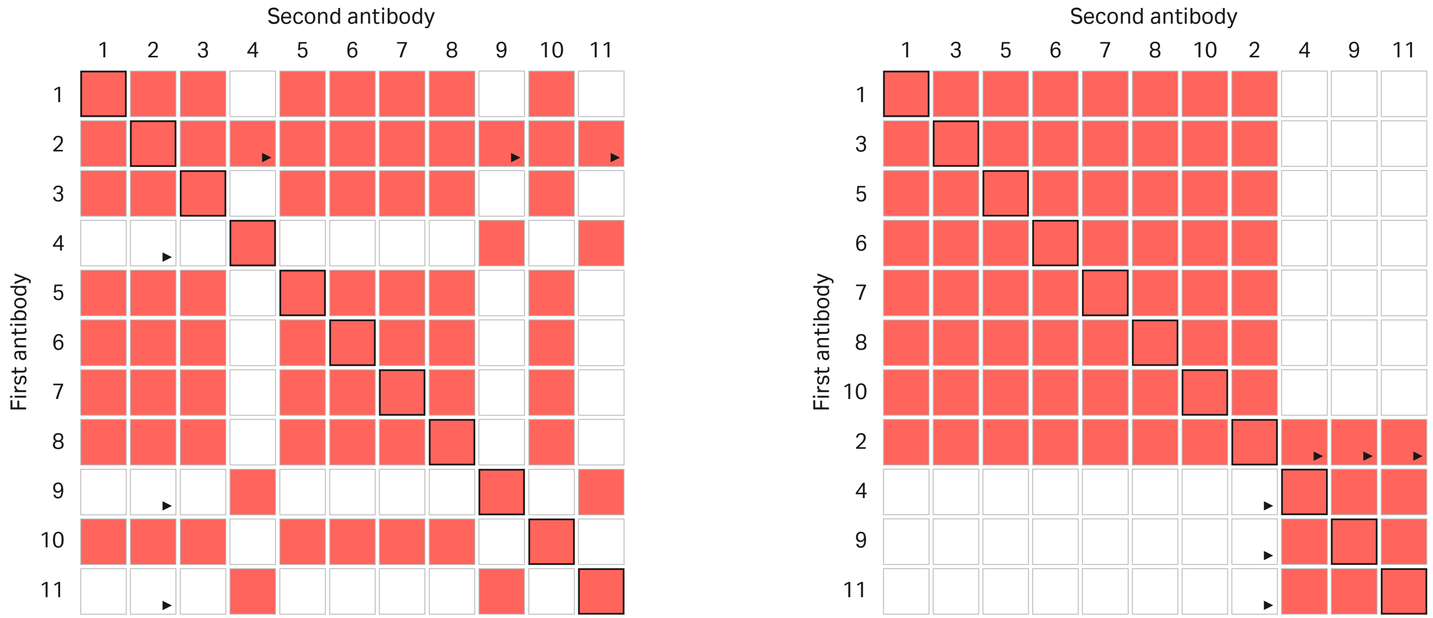

Fig 5. Epitope binning results presented in a heat map. The heat map to the left is sorted by run order which can make it difficult to identify binding patterns. By sorting by bins (heat map to the right) binding patterns and bins are more easily identified as areas along the diagonal of the heat map.

Working with the bin chart

To further empower assessment and displaying of epitope binning result you can use the bin chart. The bin chart typically represents the different bins as graphical clusters which often have connectors to illustrate relationships and overlaps between bins. A cluster without connectors illustrates an independent bin, consisting of antibodies with a common and likely unique epitope on the antigen. An example of a bin chart is shown in Figure 6.

There are many styles and variants of bin charts in the field. In Biacore™ Insight Epitope Binning Extension, the bin chart is a circle with a unique color for each antibody segment (Fig 6). Each bin has a number and unique color fusing the antibody segments together. Connector lines illustrate interactions between bins. A smooth line indicates mutual interactions and a line with an arrow on it indicates unidirectional interactions with the arrow pointing in the blocking direction. There are no connectors to or from separate bins since there are no interactions between these bins. Separate bins also have gaps on both sides.

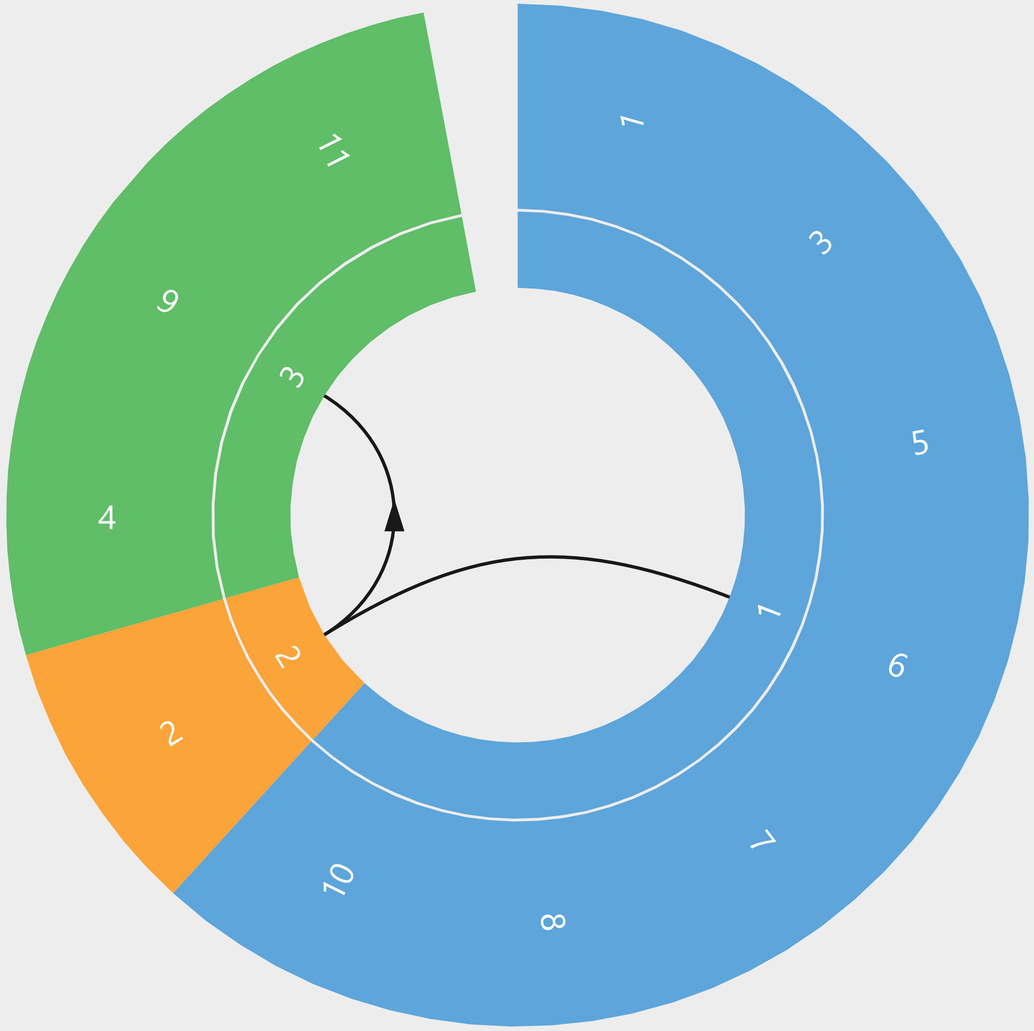

Fig 6. This figure shows the binning results from the heat map in the section above represented in a bin chart. There are three bins, i.e., antibodies with an identical or unique blocking pattern. Antibodies 1, 3, 5, 6, 7, 8, 10 constitute bin 1 that have a mutual blocking with antibody 2 in bin 2. Antibody 2 cannot belong to bin 1 since it also blocks antibodies 4, 9 and 11 in bin 3. Antibody 2 ends up alone in bin 2. There are no separate bins in this example.

Case study: Data grooming by first antibody

Epitope binning evaluations focus mainly on the assay component that is used for evaluation, i.e., the second antibody injection for sandwich and tandem assays and the antigen/second antibody injection for premix assays. To really know your reagents, assay, and verify results you need to examine all components of the assay.

Evaluation is not always clear-cut and sometimes data needs to be scrutinized and examined in detail to fully understand the results. In this section we look at a real-life data set with poor resolution between blockers and non-blockers. We also go over how the reason for the poor resolution was identified and corrected.

Poor resolution between blockers and non-blockers

This data set was run in the sandwich assay format. When examining the sensorgram overlay of the second antibody injections we can see that there is no gap between blocking and non-blocking responses. This makes it very difficult to set cut-offs and identify bins in the heat map and bin chart (Fig 7). As a first step the behavior of all first antibodies was examined.

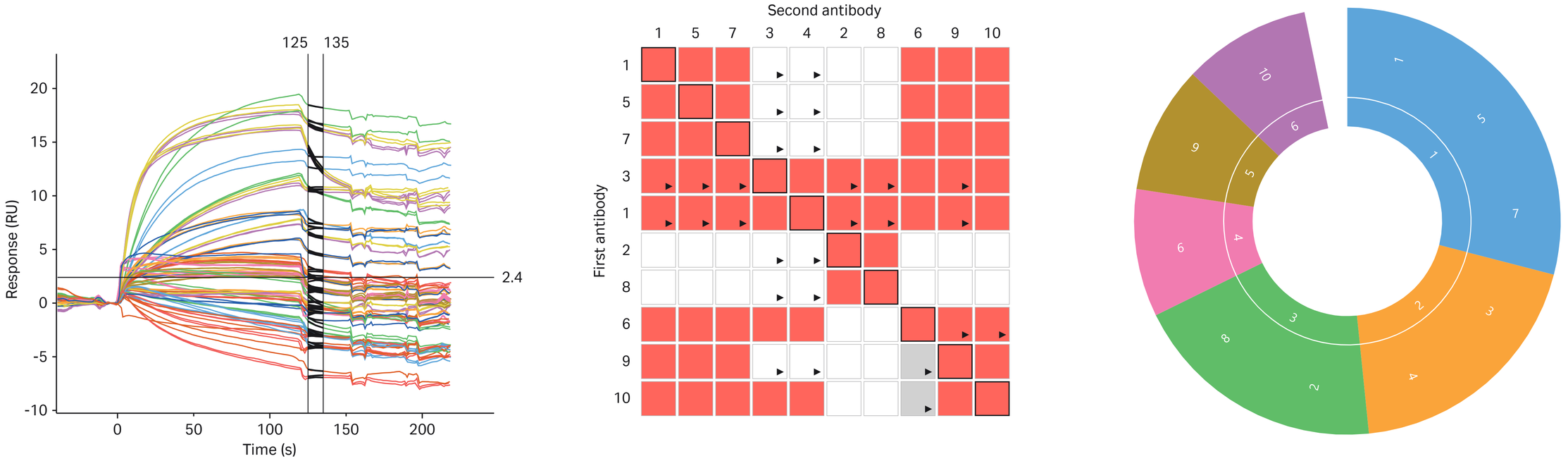

Fig 7. Example of an epitope binning experiment. With all sensorgrams in the run included, there is no gap between blocking and non-blocking response. Thus, it is not possible to set cut-offs and obtain an accurate binning classification.

Low antigen binding

To identify a possible reason for the poor resolution, overlay charts of all sensorgrams with a common first antibody were created, one first antibody at a time (Fig 8). It was then revealed that two of the antibodies in the study had very low antigen binding when used as first antibody. This resulted in a very low second antibody binding and no resolution between blockers and non-blockers.

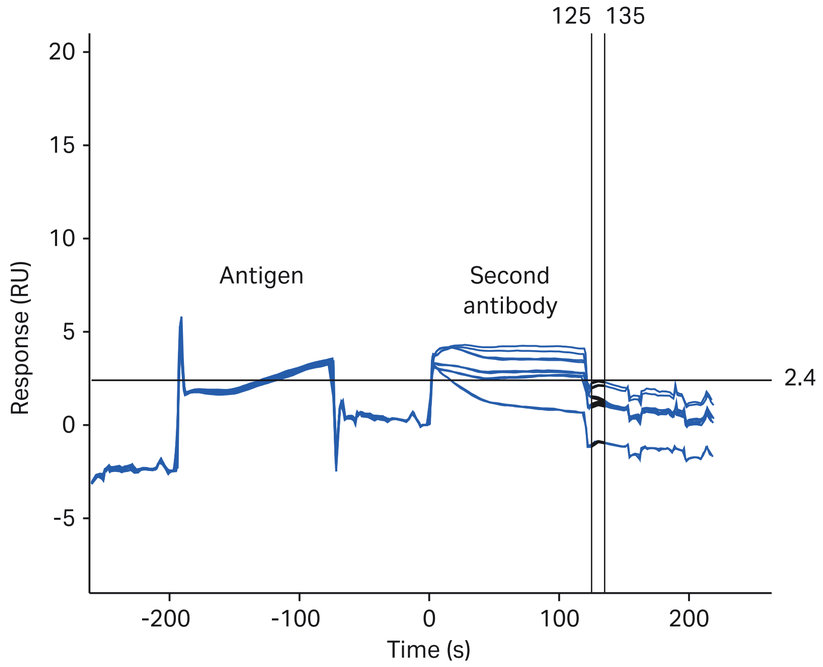

Fig 8. Different second antibody binding sensorgrams with a common first antibody. Two of the antibodies included the study had very low antigen binding when used as first antibody which resulted in low second antibody binding and no resolution between blockers and non-blockers.

Excluding low binding antibodies

The antibodies with low antigen binding were excluded from the evaluation which resulted in improved resolution between blocking and non-blocking response. The cut-off still had to remain below the visually apparent gap (Fig 9).

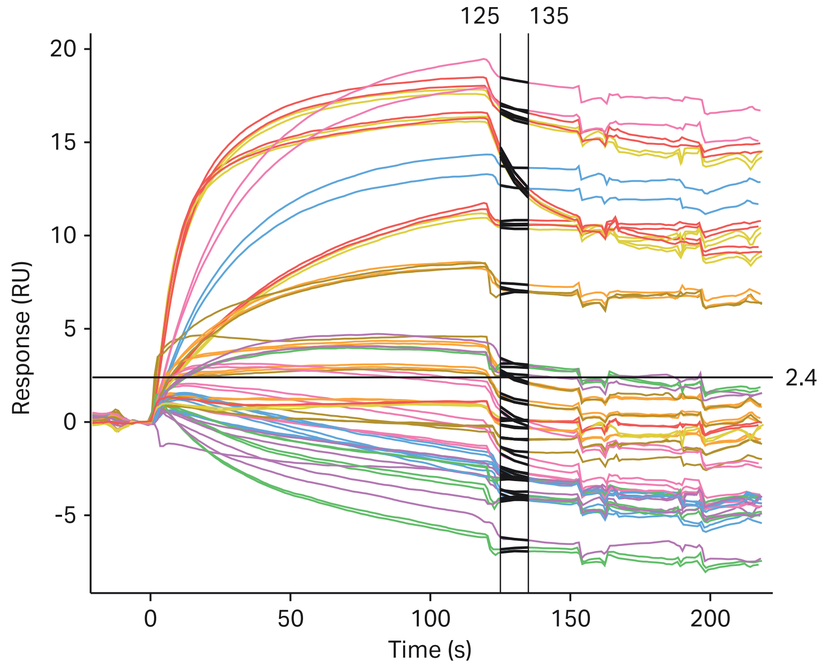

Fig 9. All remaining sensorgrams in the evaluation colored by first antibody. By excluding the antibodies with low antigen binding from the evaluation the resolution between blocking and non-blocking pairs were improved, which can be seen by comparing with the non-existent gap in the sensorgram overlay in Figure 7.

Varying drift pattern between first antibodies

When the sensorgrams were examined in more detail it was revealed that one of the first antibodies caused a downward drift throughout all second antibody injections (Fig 10). The resolution between blocking and non-blocking second antibody responses for this first antibody was good. The non-blocking responses of this first antibody were very close to the blocking responses for the other first antibodies in the run, making the cut-off placement more subtle. By placing the cut-off low in the general gap between blockers and non-blockers it was possible to keep this data in the evaluation.

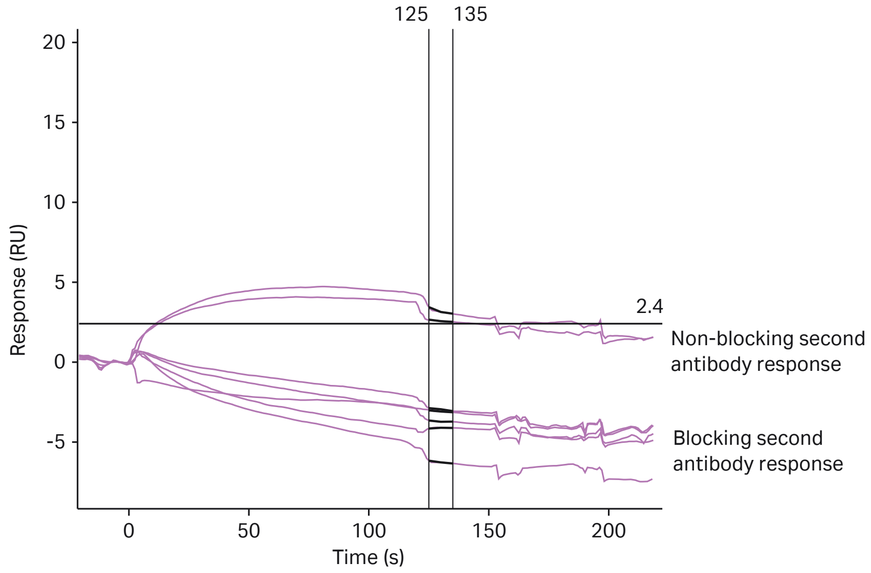

Fig 10. Different second antibody binding sensorgrams with a common first antibody. This first antibody caused a drift in all subsequent second antibody injections which resulted in non-blocking responses (above cut-off) being close to blocking responses of second antibodies with other first antibodies (Fig 9). This resulted in a narrow range with respect to lower cut-off placement. 2.4 RU was considered adequate.

Final results

Removal of disturbing antibodies and a precise placement of cut-offs in the sensorgram overlay shown in Figure 9, resulted in a very clear and easy to interpret binning result (Fig 11). The grey squares in the heat map represent sensorgrams that were excluded due to disturbances during the second antibody injection. Unidirectional binding is indicated by the arrowhead. By including unidirectional blocking in the binning definition the result is two well defined bins for the eight remaining antibodies.

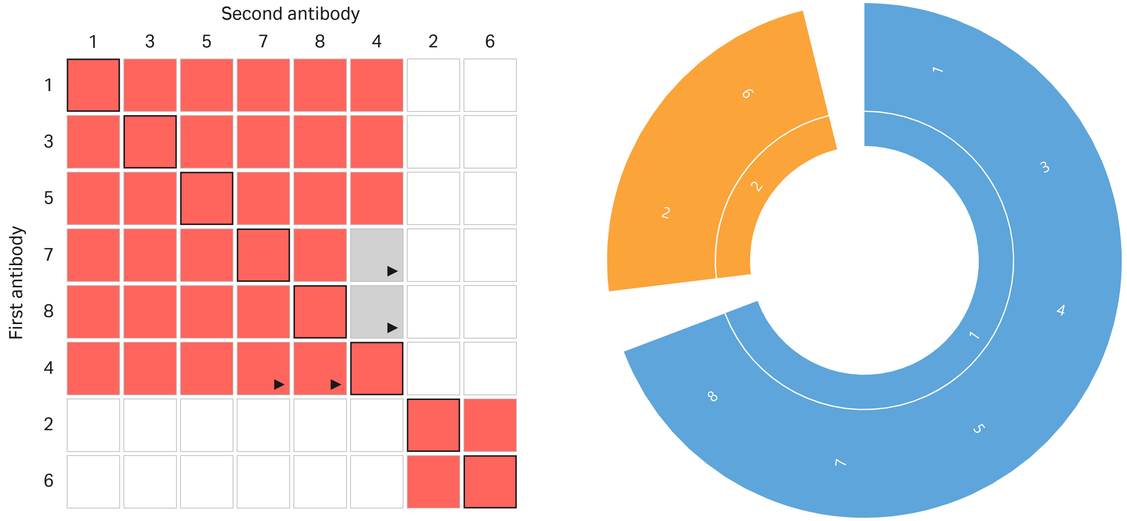

Fig 11. Result of the epitope binning after removal of disturbing antibodies and adjustment of cut-offs. Two well defined bins are identified for the eight antibodies.

Two conclusions can be drawn from this case study.

- Looking at the sensorgram overlays by first antibody revealed that binning of the two excluded antibodies could not be done in this run. Keeping them in the evaluation would have obscured the binning pattern of the other antibodies. By excluding them, the main part of the run could be reliably evaluated.
- The necessity to place the cut-off very low was revealed and justified.

By taking actions on these two issues the quality of the data set was improved and a clear-cut epitope binning result was obtained.

References

1. The Therapeutic Monoclonal Antibody Product Market, Ecker *et al.*, Bioprocess International, October 30, 2020

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For local office contact information, visit cytiva.com/contact

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