Best Practices for Designing and Analyzing Epitope Binning Experiments Using the Carterra LSA and Epitope Software

Dan Bedinger, Ph.D.

Applications Science Team Lead

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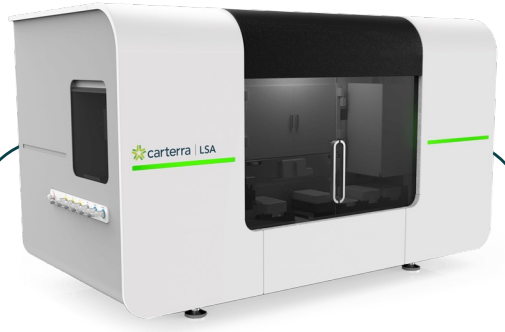
The LSA is a complete HT-SPR platform for biologics discovery and screening.



LSA instrument

Control and analysis software

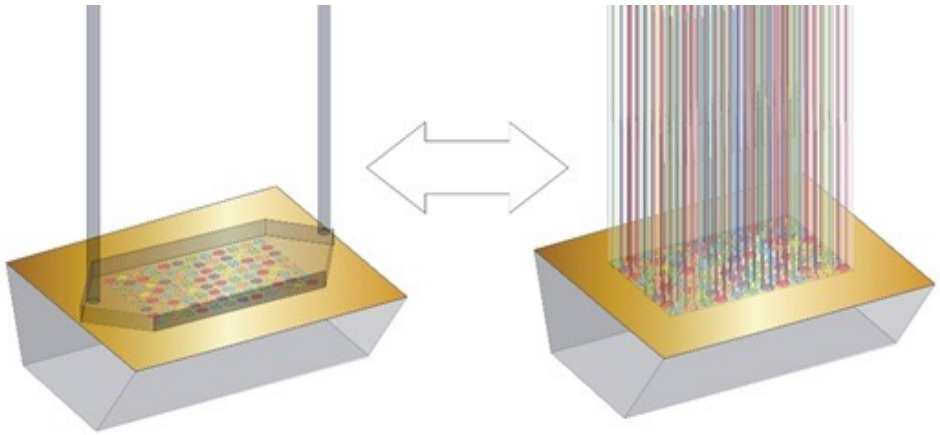
Biosensor chips and consumables

LSA integrates flow printing & array SPR.

Single-channel mode
(single flow cell)

Multi-channel mode
(96-channel printhead)

Minimal analyte consumption via "one-on-many" assay format

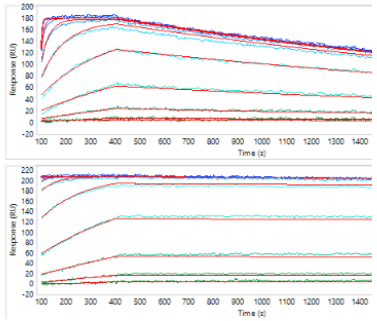


Serially print at 4 nested locations to create a 384-array

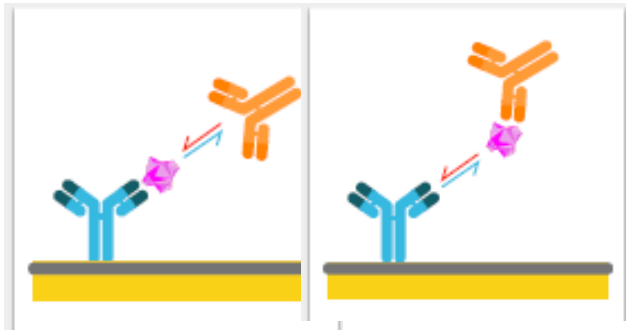
- Automated flow cell switching between multi- and single-channel modes
- 384 reaction spots + reference interspots per array
- In-line reloading of array
- Supports capture formats and standard amine coupling

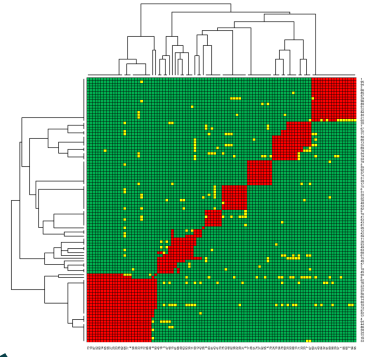
LSA's Core Applications

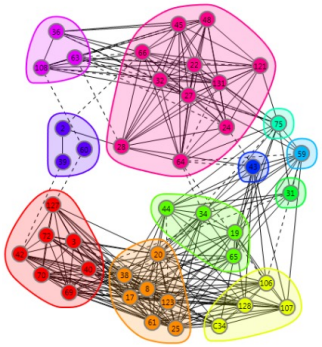
Kinetics/Affinity



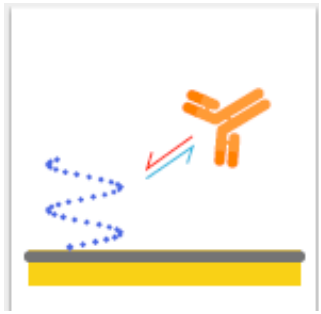
Epitope Binning

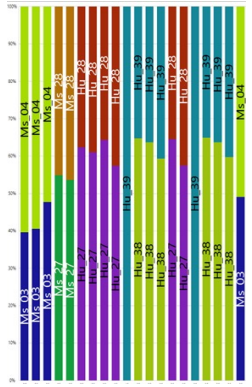




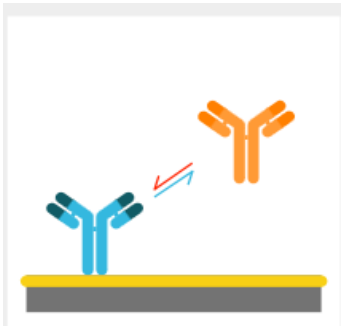


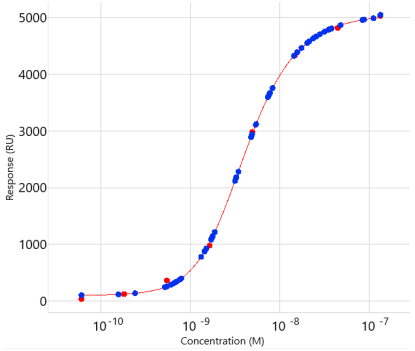
Mapping





Quantitation



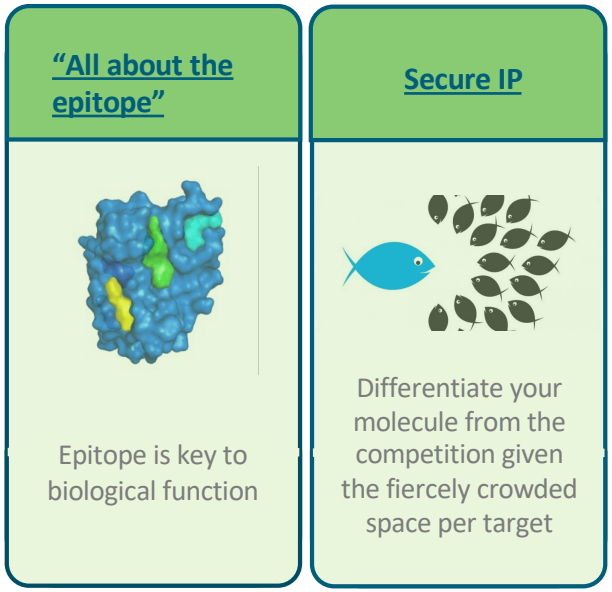


Topics

- Why epitope binning?
- Carterra's assay strategies
- Epitope software analysis overview
- Experimental considerations and assay design
- Analysis strategies
  - Data curation
  - Creating community plots

Why Competitive Epitope Binning?

- The functionality and MOA of a therapeutic antibody is linked to its epitope
- Currently affinity can be readily assessed, engineered, and optimized
- Epitope binding is innate to a mAb and can not be readily engineered and must be screened or selected
- Early epitope characterization can serve as a surrogate for functional diversity
- Inform large sequence sets to functional classes
- Establish IP and demonstrate differentiation from other molecules



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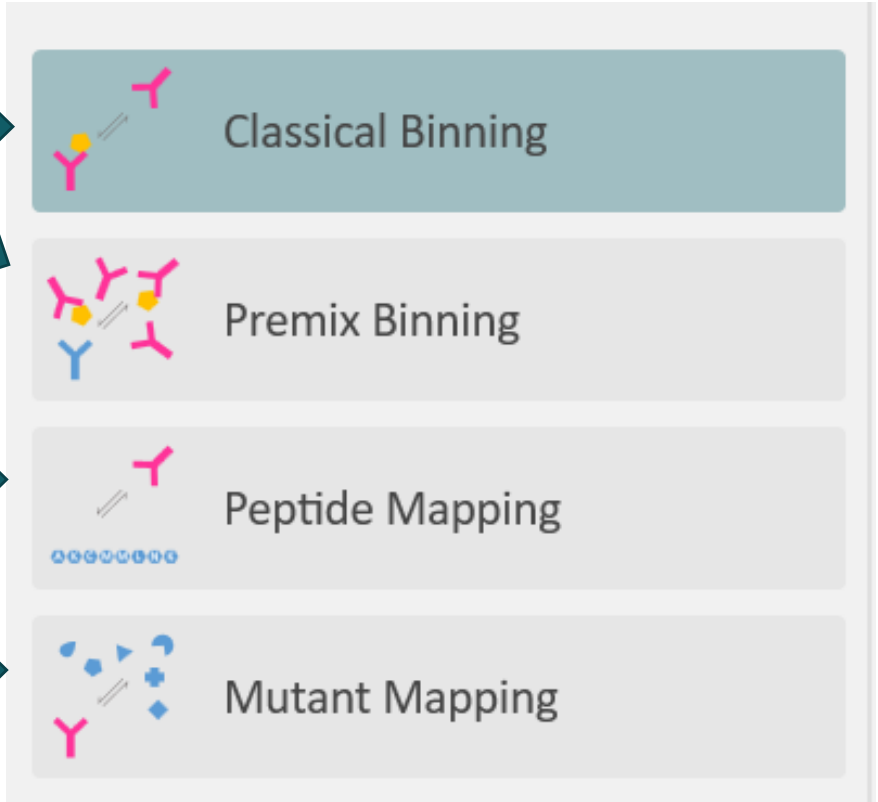
Binning is Just One Way to Characterize Epitope Using the LSA

Competitive binning: Determine if two mAbs can bind simultaneously to the same antigen

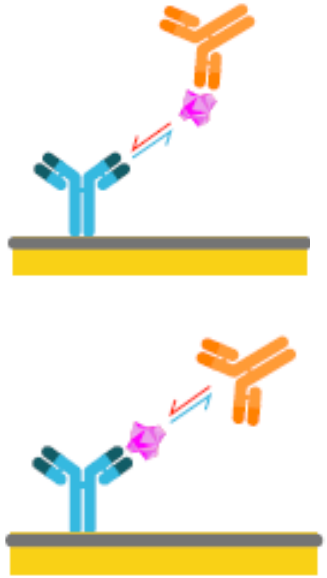
Epitope mapping: Identify residues on antigen peptides that mAbs bind

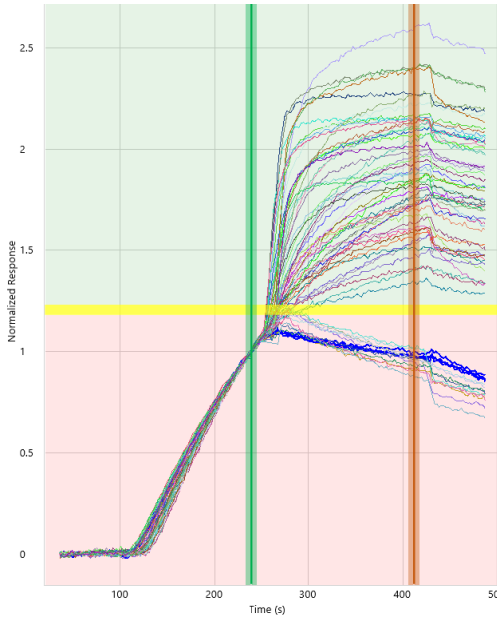
Mutant mapping: Use antigen mutants to determine what residues/regions mAbs recognize

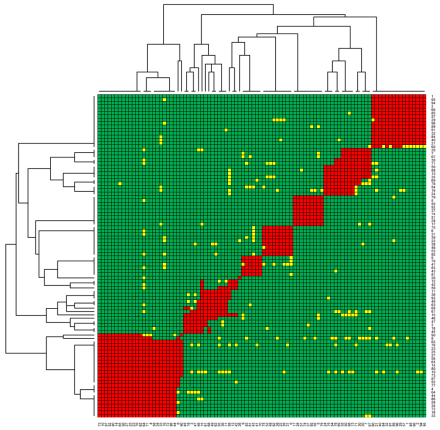


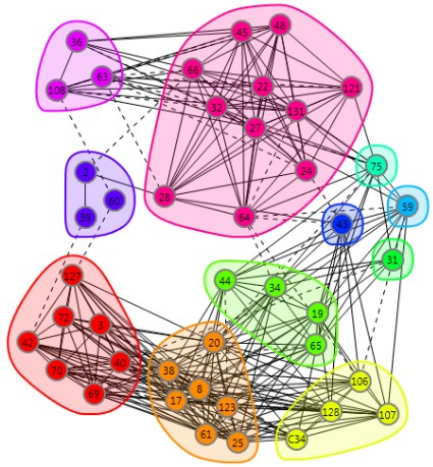
LSA Epitope Binning Workflow

Compete Clones → Define Competitive Thresholds → Represent Epitope Relationships using Heat Maps, Network Plots, etc.

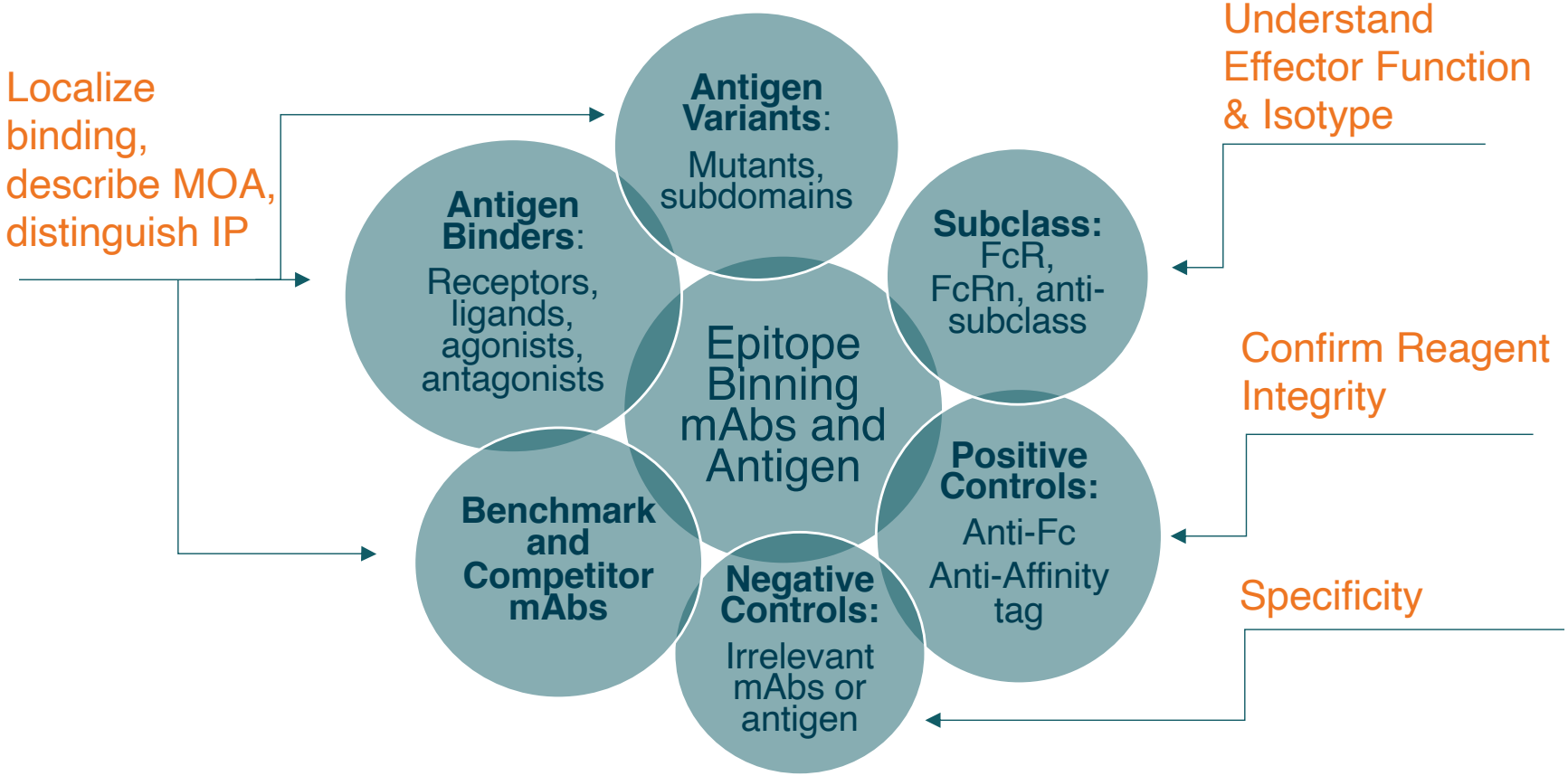








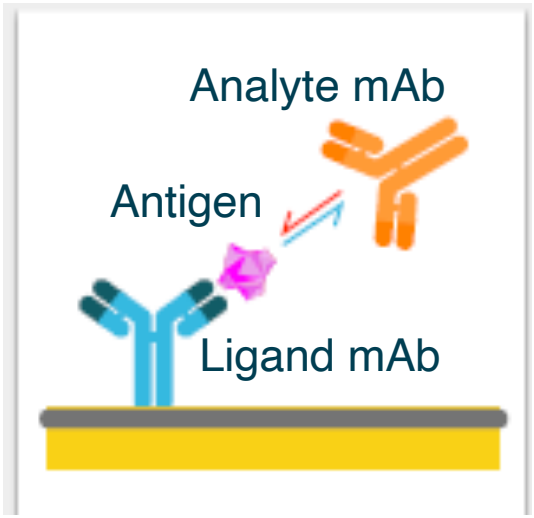
Supplement the Binning Assay with Additional Reagents



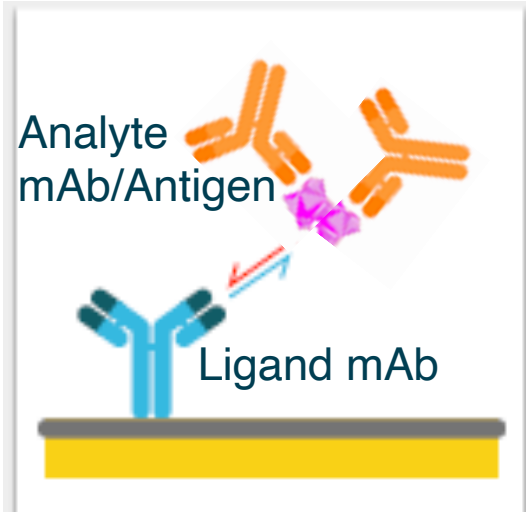
- These approaches take advantage of additional capacity within the array and use the analyte injections to expand the information from the assay - FREE DATA

Antigen Valency Dictates Epitope Binning Assay Format

Two formats best suited for the “One-On-Many” design of the LSA

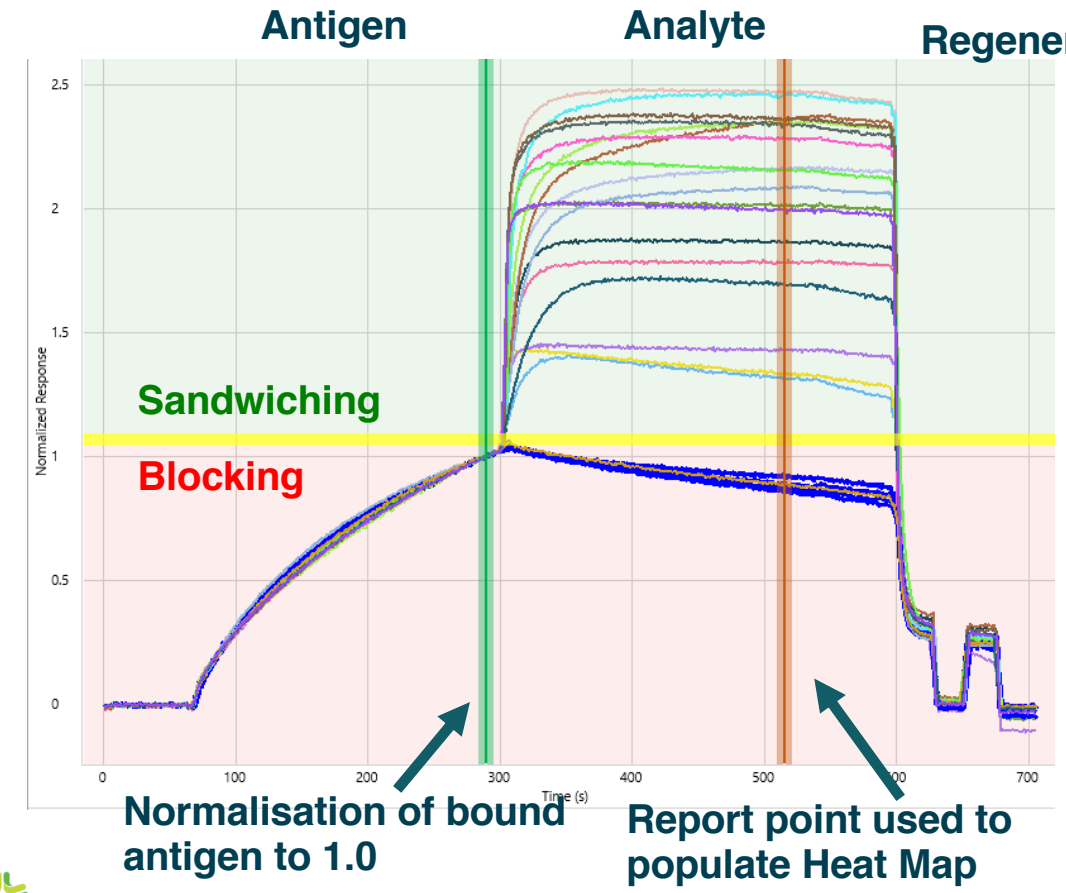


Monovalent Antigen: Classical Binning



Multivalent Antigen: Premix Binning

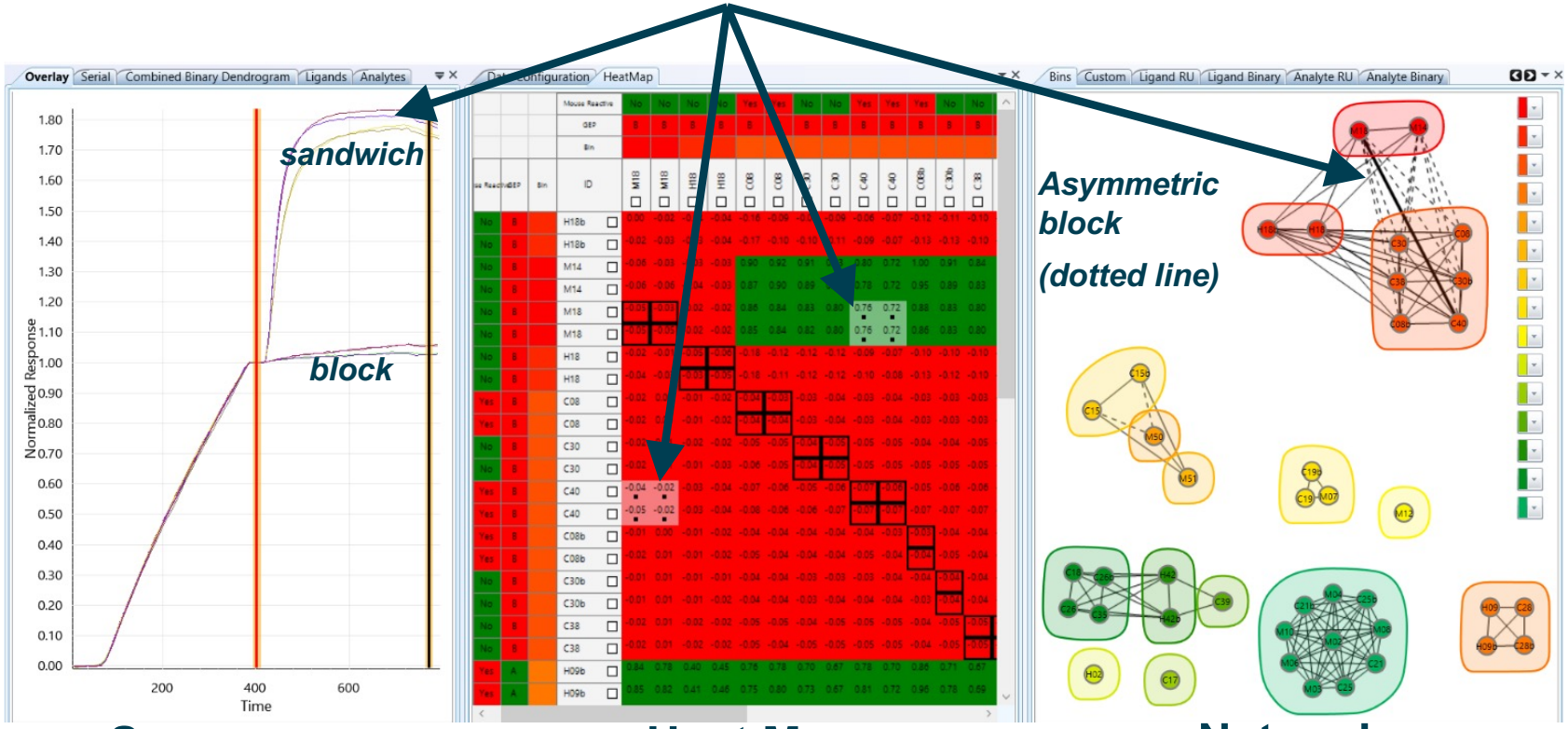
The Classical Sandwich Binning Cycle



1. Each cycle starts with baseline phase
2. Followed by the antigen injection
3. Which is then immediately followed by Ab injection.
4. Cycle is ended with acidic regeneration & stabilisation.

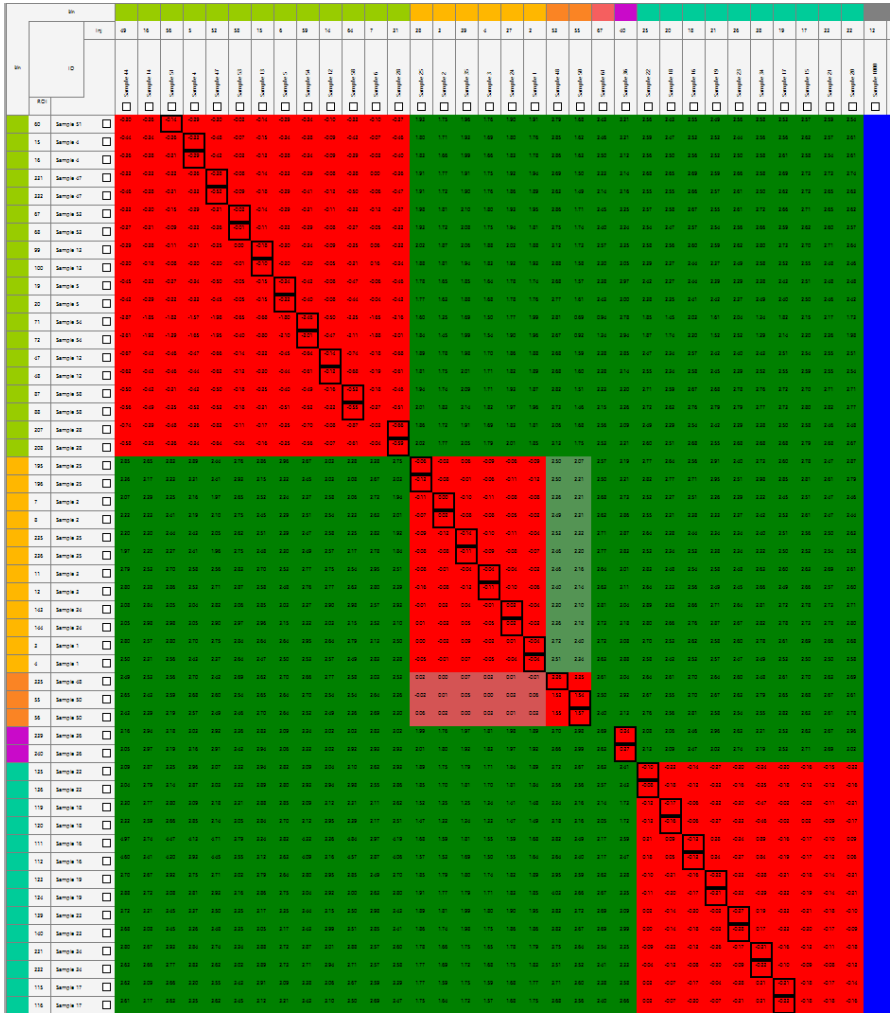
Epitope Binning Software User Interface

Data Linked Across Three Visualization Panels



Sorted Heat Map (Competition Matrix)

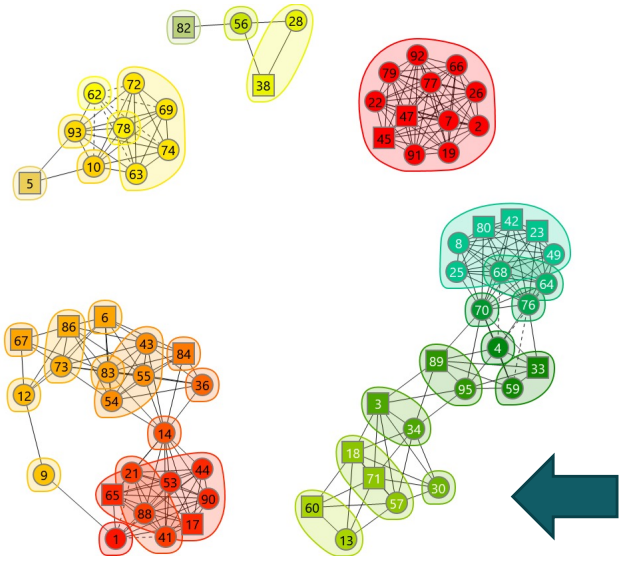
- The Heat Map shows all of competition information in analysis
- Ligands are shown as rows and analyte injections as columns
- Red indicates competition, Green a sandwich
- Self versus self are black outlined
- Highlighted are asymmetrical competition profiles
- Software automatically sorts clones together based on shared competition profiles in this plot

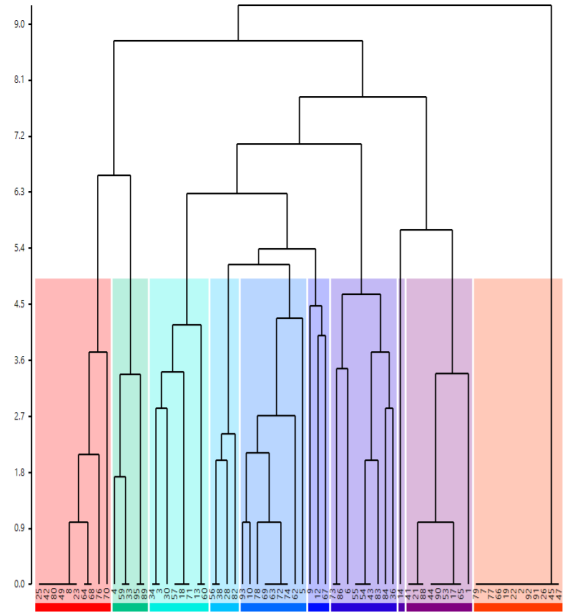


Flexible Exploration of Epitope Clusters

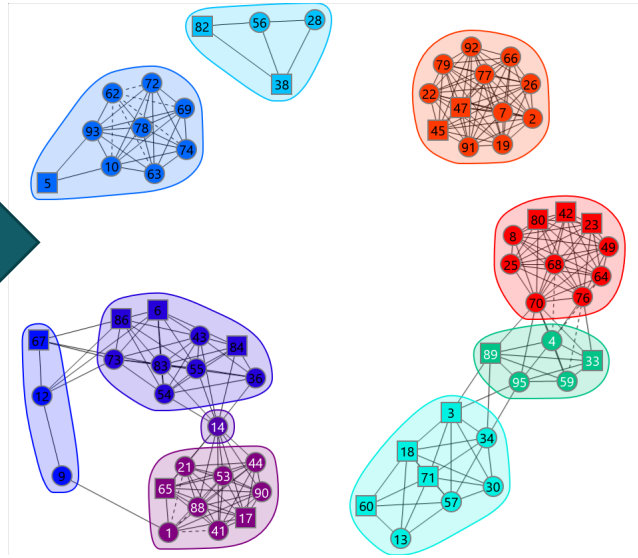
- Lines (chords) represent blocking; distance typically represents degree of epitope similarity
- Visualize competitive relationships dynamically using hierarchical clustering
- Community view aids interpretation of clusters with partial epitope overlaps

Objective Groupings:
Bin-Level Network



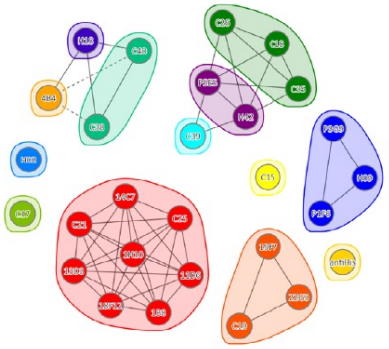


Subjective Groupings:
Community-Level Network



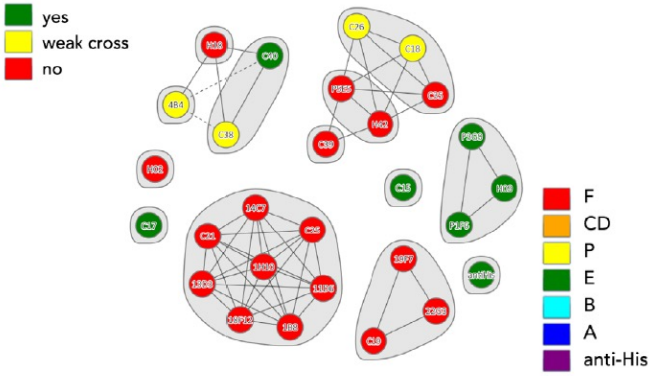
Inform and Refine Lead Selection with Orthogonal Data

Bin

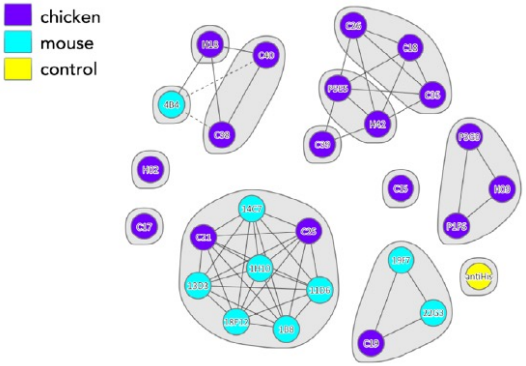


Import orthogonal data as a pasted column of descriptors

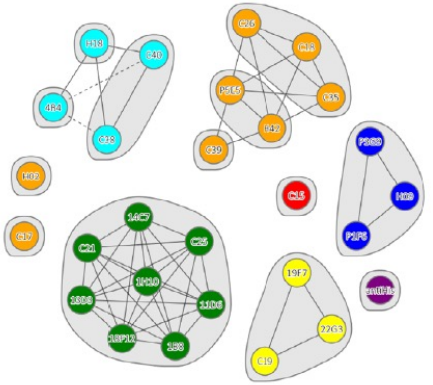
Mouse Cross-reactivity



Library



Mapping



See Carterra Epitope Diversity App Note

Getting Good Data: Epitope Binning Methods

Data quality is essential- bin map fidelity is dependent on the reliability of sandwich/block calls

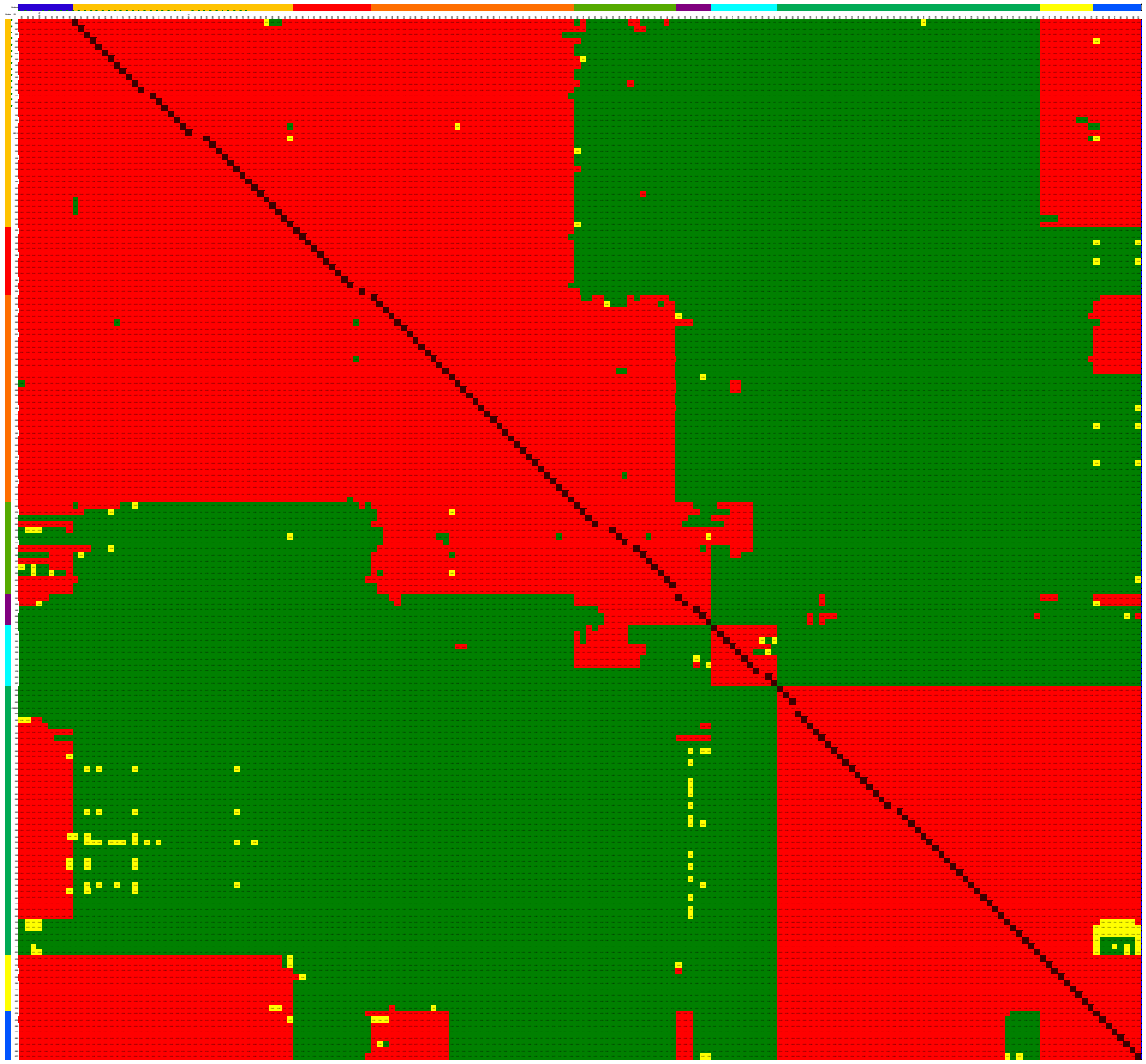
- Immobilization
- Selection of antigen and analyte concentrations
- Optimization of regeneration conditions
- Appropriate data curation
  - Low quality signals add little value and significant complexity
- Appropriate use of dendrogram and community clustering

Larger and More Diverse Binning Sets Add Resolution

- Large heat maps provide many pair-wise interactions
  - 96 x 96 = 9,216 interactions
  - 192 x 192 = 36,824 interactions
  - 384 x 384 = 147,456 interactions
- Each unique interaction can be thought of as a probe
  - Each unique sample has the potential to elucidate new behaviors or subtle differences present in the epitope
  - The more diverse set you have, the more resolution you get
- In addition to epitope resolution, large scale binning allows for a more powerful curation of the data: You can tell what is consistent and what is not
  - The impact of minor imperfections in data quality (a few questionable ligand or analyte responses) can easily be compensated for in the community plots

Example Heat Map

- 170 ligands x 188 analytes 31,960 interactions
- Currently clustered as 10 communities
- Binning map is highly symmetric
- All ligands show self-versus self blockade



Immobilization

- High binding signals are good
  - Unlike kinetics, a large signal in epitope binning is beneficial and serves to separate the real signal from background effects or potential complexities
  - Surface transport characteristics are generally not a concern
  - For most systems an HC3OM (linear polycarboxylate) gives adequate signal
- Covalent immobilization (or near covalent) is typically required
  - Techniques include direct immobilization, biotinylation/streptavidin linkage, or capture then cross-link approaches
- Immobilization strategy needs to preserve maximal ligand activity

Antigen Concentration Considerations

- Antigen binding is a key step in epitope binning and is used for normalization of all sensorgrams
  - Inject an antigen concentration series over the array to scout
- For classical sandwich you want robust loading of antigen for all ligands
  - Higher is OK, optimization is primarily around reagent use minimization rather than assay performance
- For Pre-mix binning you want to find the minimal concentration which provides a usable signal, as the analyte mAbs must compete
  - High antigen concentrations in pre-mix discourage complete competition and can make the data harder to interpret

Analyte mAb Concentrations

- For classical sandwich assays, analyte binding rates are determined by the kinetics (k_a and K_D) and the concentration of the analyte
  - Lower affinity analytes need higher concentrations to drive sandwiching
  - Using higher analyte concentrations for panels with varied affinities will simplify interpretation of the results
  - For IgGs 20 to 50 µg/mL (200 to 333 nM) is a good range
- For pre-mix binning, analyte mAb concentration needs to be:
  - Well in excess of the K_D of the mAb:Ag interaction
  - In stoichiometric excess of the antigen
  - For diverse panels, 50 µg/mL IgG (333 nM) is a good target

Regen Optimization - Effective Regeneration is Essential

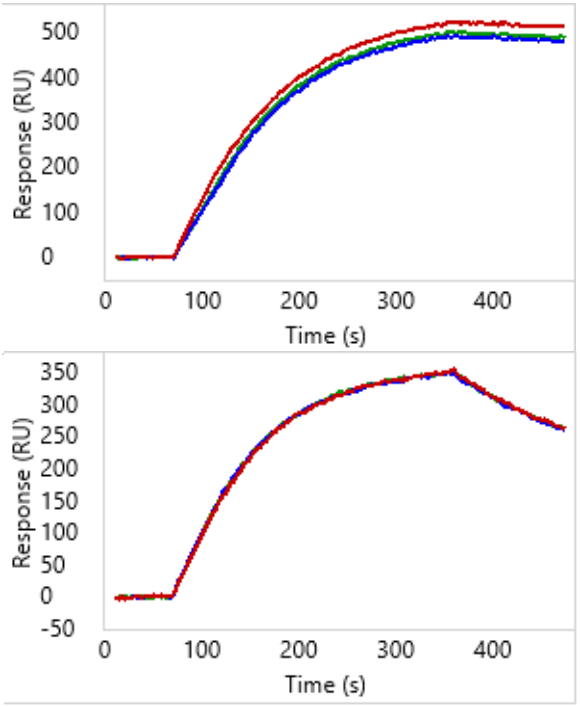
- Verify regen conditions prior to starting a binning assay
- Most Ab antigen interactions will regenerate with low pH solutions
  - Pierce IgG elution buffer pH 2.8 + 1 M NaCl (30 to 90 seconds)
    - Good for many kinetics systems, but not strong enough for most binning assays
  - **10 mM Glycine pH 2.0 (10-30 seconds)**
    - Common successful solution, some acid sensitive clones will likely lose activity
  - H_3PO_4 (0.425 to 0.85%)- (10-30 seconds)
    - Better regeneration with more ligand attrition
- Other strategies- when acid is not working
  - **pH 11.0** - 50mM NaOH + 1M NaCl + 0.05% Tween (pH down with H_3PO_4)
  - 3M $MgCl_2$ will work on some systems and is a weak chaotropic salt

Visualization of Regeneration Effectiveness – Overlay View

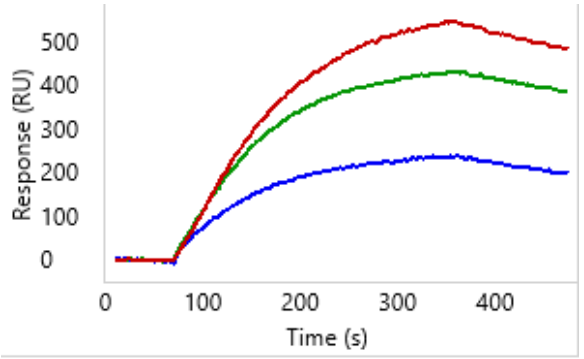
First Cycle
Second Cycle
Third Cycle

- Three identical antigen binding cycles are shown
- Ligand performance is assessed for:
  - completeness of regeneration- does the signal return to baseline
  - Reproducibility of binding- is the same level achieved for each cycle

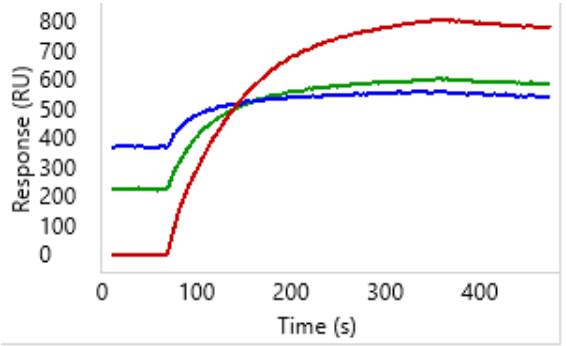
proper regeneration



loss of activity



incomplete regeneration



Effective Processing and Curation of the Data is Critical

Data curation steps:

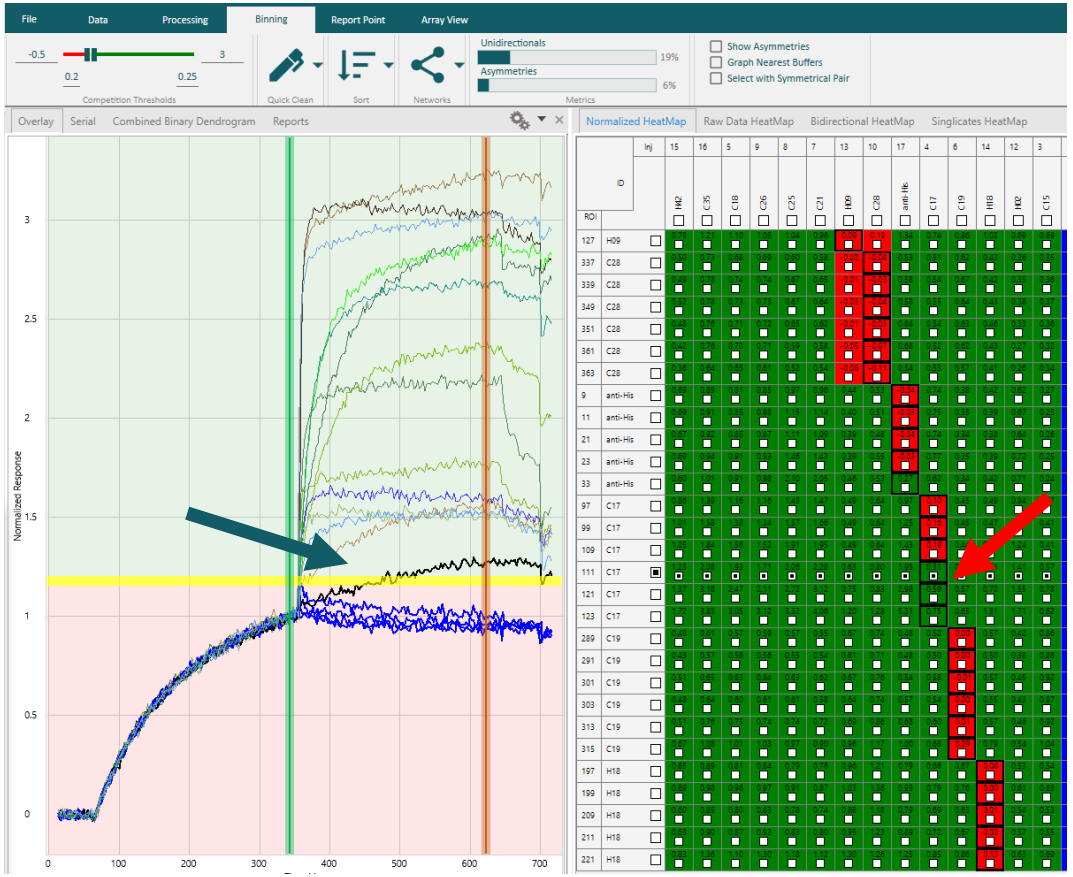
- Apply referencing, cropping, and y-alignment
- Exclude non-binding or problematic ligands
- Exclude non-binding analytes
  - Non-binder clones and negative controls do not fit blocking/sandwiching paradigm and should be excluded from the final analysis
  - In pre-mix assays scrutinize analytes with many or only sandwiching relationships
  - In classical binning, scrutinize analytes with entirely blocking responses
  - These reflect low or no analyte activity and complicate interpretation
- Data can then be sorted and interrogated for other features;
  - Self-sandwiching
  - Asymmetrical competition
  - Cluster/pattern outliers

Ligand Assessment

- Use array view to easily view all ligand for activity
- Note ligands with low or no activity or near total loss of binding over the course of the assay
- Exclude these ligands by unchecking on the main data page

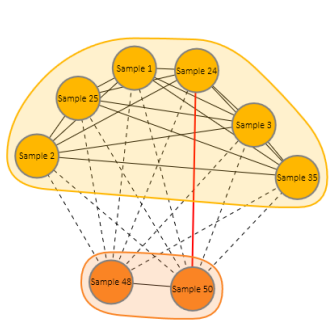
Look for self-versus-self Sandwichers

- Next look for self-self interactions which are not marked as blockers
- This can either highlight that the cut-offs are not optimized or if there are ligands with complex behaviors
  - Incomplete regeneration can lead to self-self signals
- In this example the ligand shows a low level of self sandwiching and adjustment of the global cutoffs can compensate

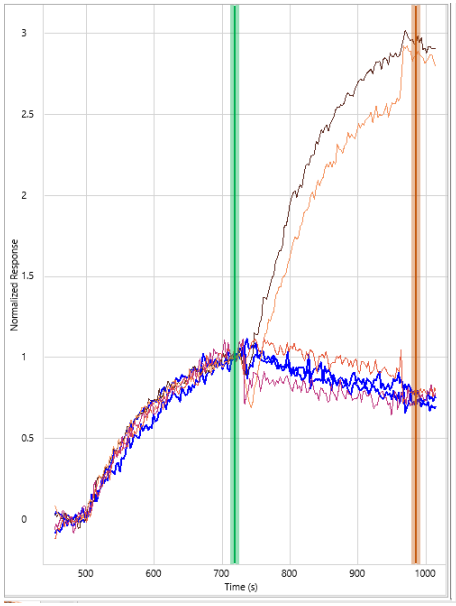


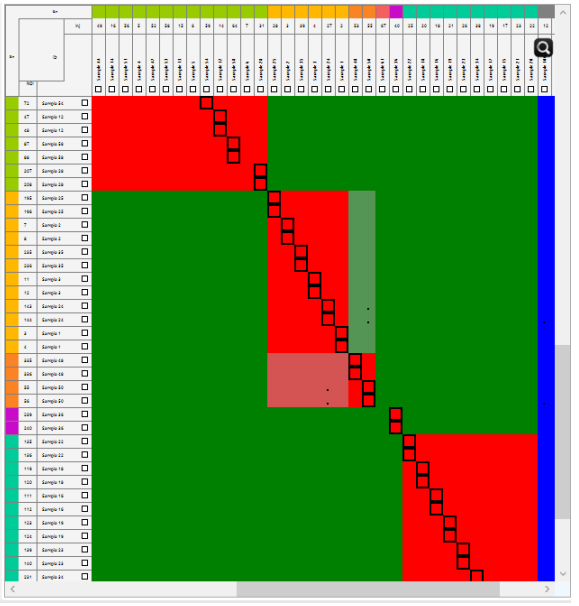
Highlight Asymmetries to Investigate Cutoff Settings

- Epitope software has a feature to highlight asymmetries, or pairs which sandwich in one orientation and not the other
- Asymmetries can be a real and important characteristic of an epitope, however it can also be a result of an improperly set cutoff



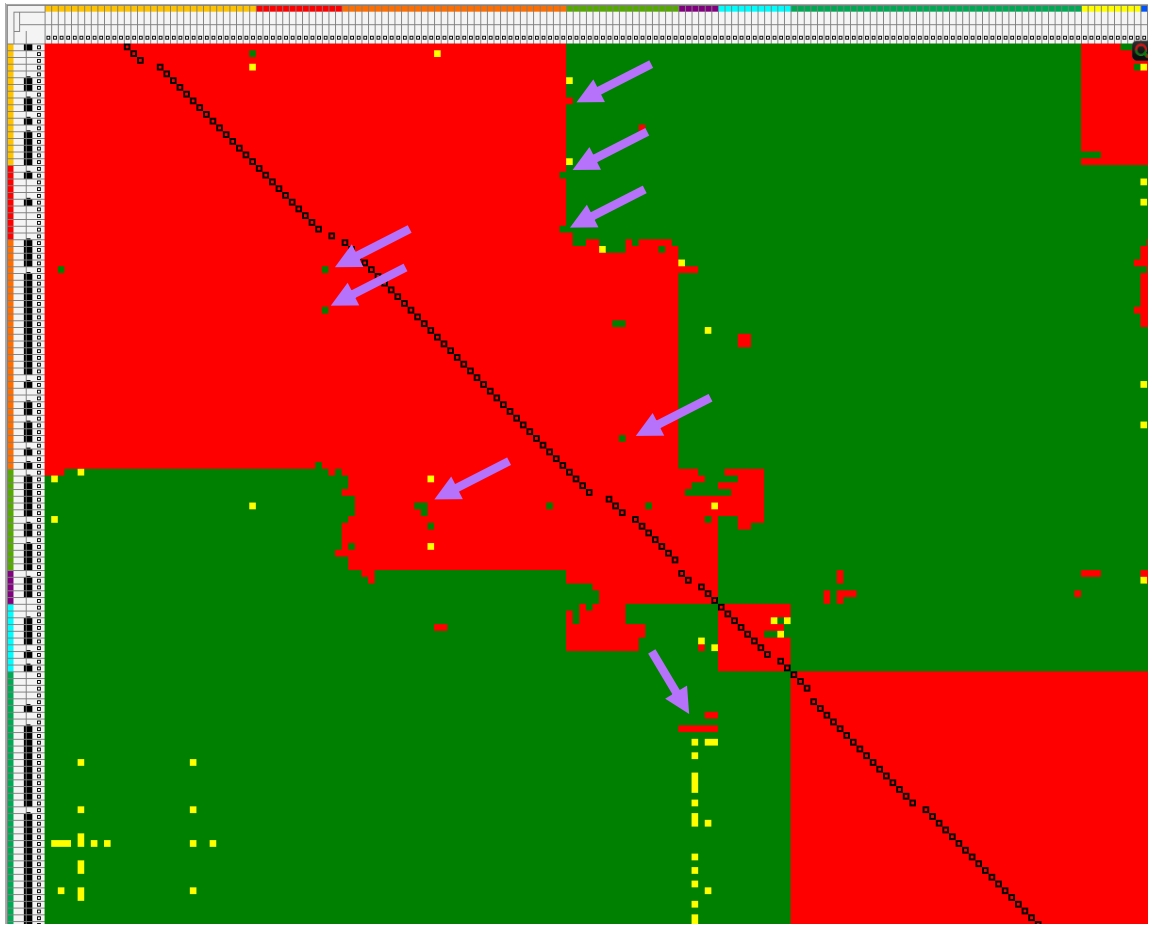
- Highlight asymmetries and then view the sensorgrams for both orientations
- If the responses are clear, leave the asymmetry
- Other times a slight shift of the cutoff may eliminate the asymmetry without effecting other aspects of the heat map. This is typically a good sign that the cut-off was causing the asymmetry, and not the biology





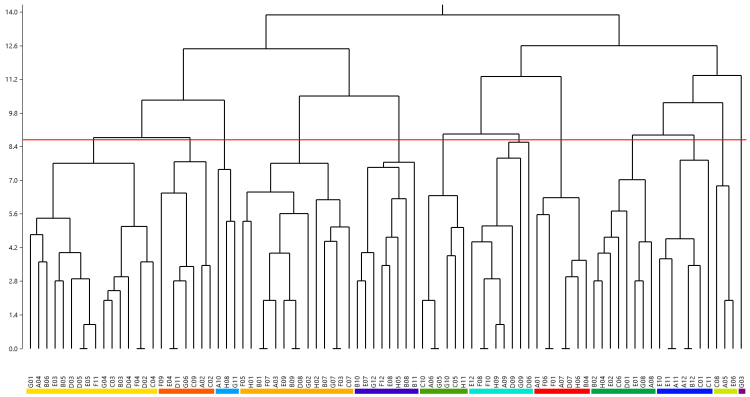
Evaluating Apparent Outlier Data Points

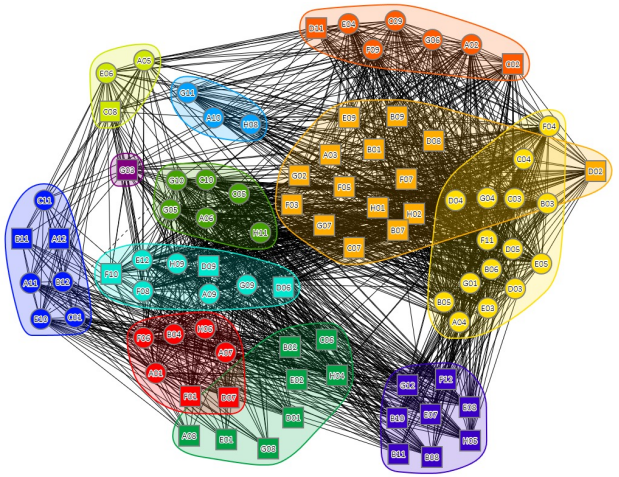
- Sufficiently rich data sets can often effectively self highlight points of questionable data
- Clear reproducible patterns emerge for conserved clustered behavior.
- Small points of deviation are present.
- Use outliers to investigate cutoffs and possible data quality issues



Community Plot and Combined Dendrogram

- Dendrogram compares similarities in both the ligand and analyte orientations
- Branch height is based on the number of differences in the blocking profile of each clone
- Clones with identical profiles are in the same bin and are shown as a flat line on the x-axis
- Cut-heights can be set to create communities





Making Community Clusters Meaningful

- All real differences can be meaningful
  - If the heatmap is 100% accurate, then only the bin level is the most biologically relevant
  - Even slight shifts in epitope can influence MOA properties
- Clustering bins is useful to map regional binding of mAbs to antigen
- Clustering allows for the grouping of clones when the data is not perfect
- **Community clusters should be set using some “rules for good communities”**

Rules for Good Communities

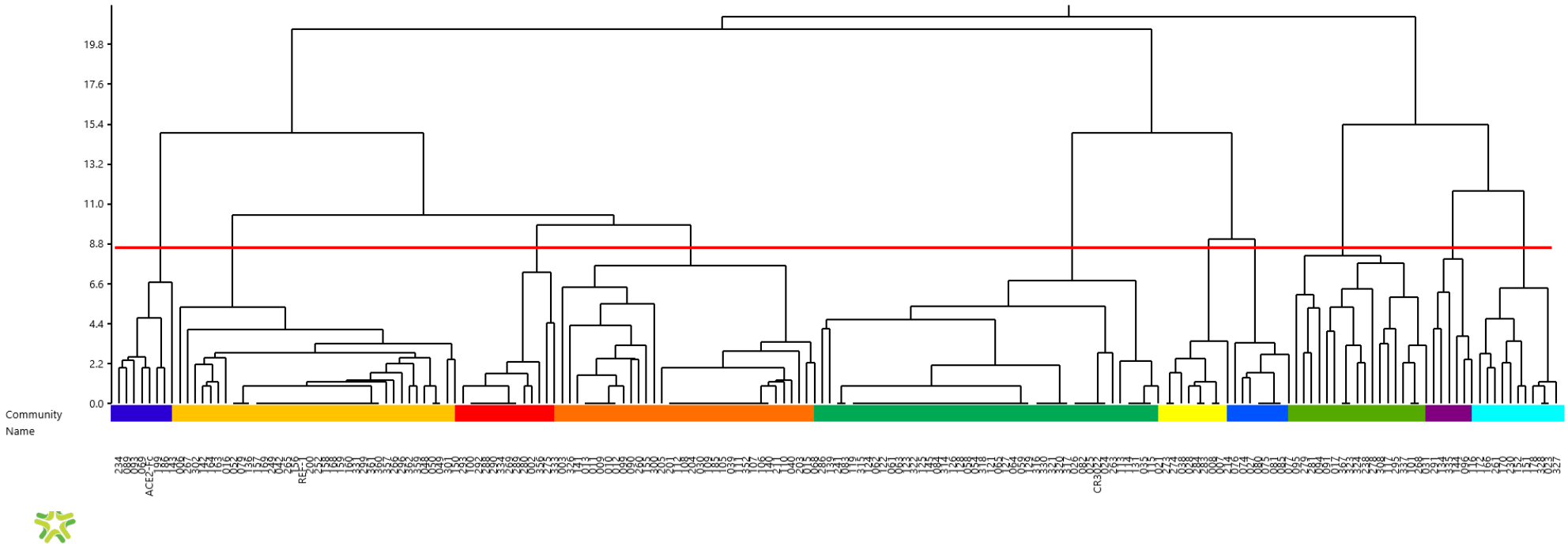
1) Clones within a community should not have sandwiching relationships with other clones in the community

2) The relationship between communities should be discreet
   - Good Example: clones in Com 1 sandwich with Com 3, 4, 5, and 7 and block Com 2 and 6
   - Bad Example: Half of the clones in Com 6 compete with Com 8
     - If this is the case, then it is necessary call out the clusters with different behaviors as sub-communities (6A, 6B) etc.

3) Some clusters may have complex relationships
   - OK, but report the complexity and possible sub-clusters

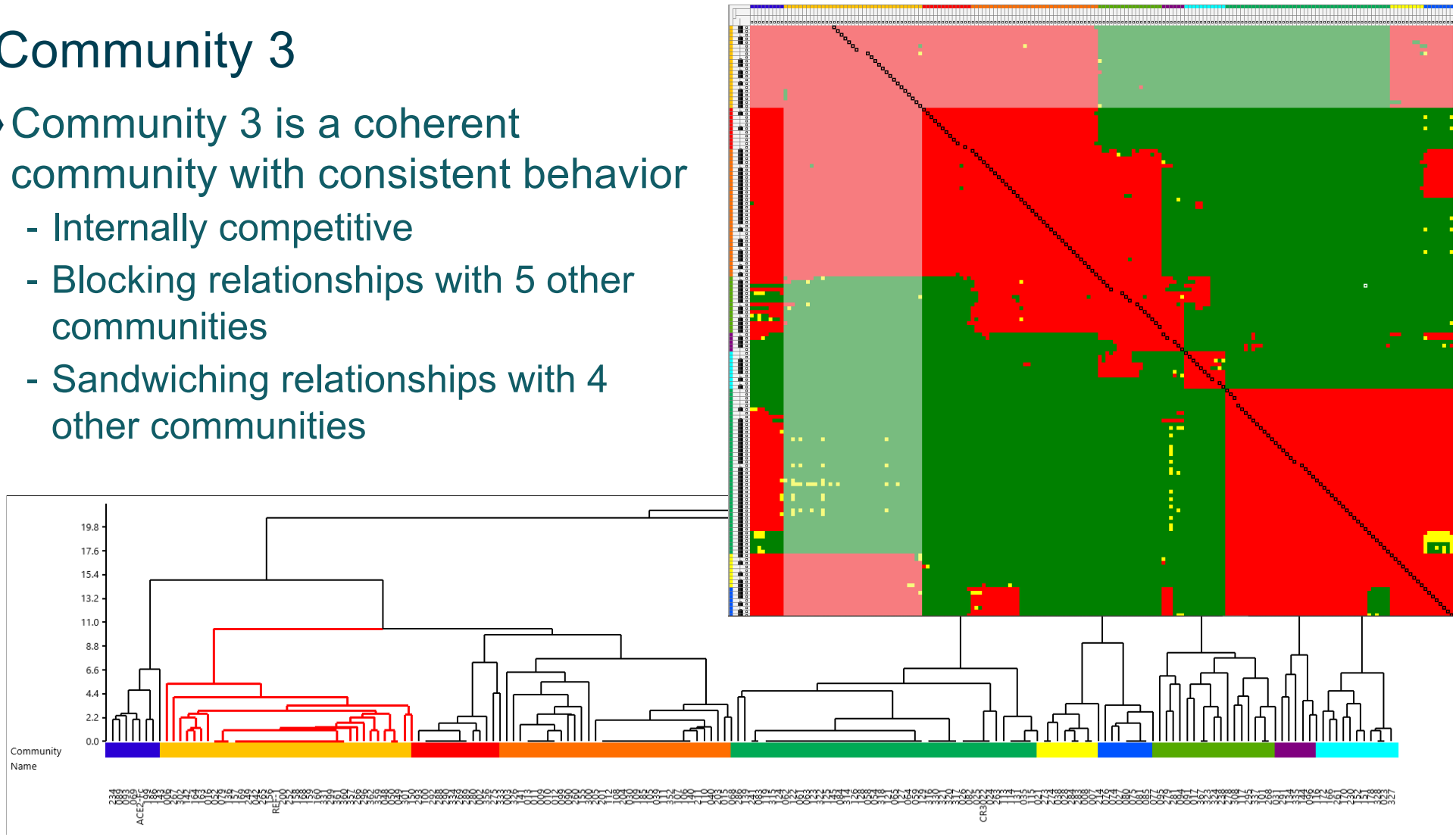
Dendrogram

- The dendrogram is shown here with a cut height creating 10 well differentiated branches
- Use heat map in Epitope software to view and evaluate clusters



Community 3

- Community 3 is a coherent community with consistent behavior
  - Internally competitive
  - Blocking relationships with 5 other communities
  - Sandwiching relationships with 4 other communities

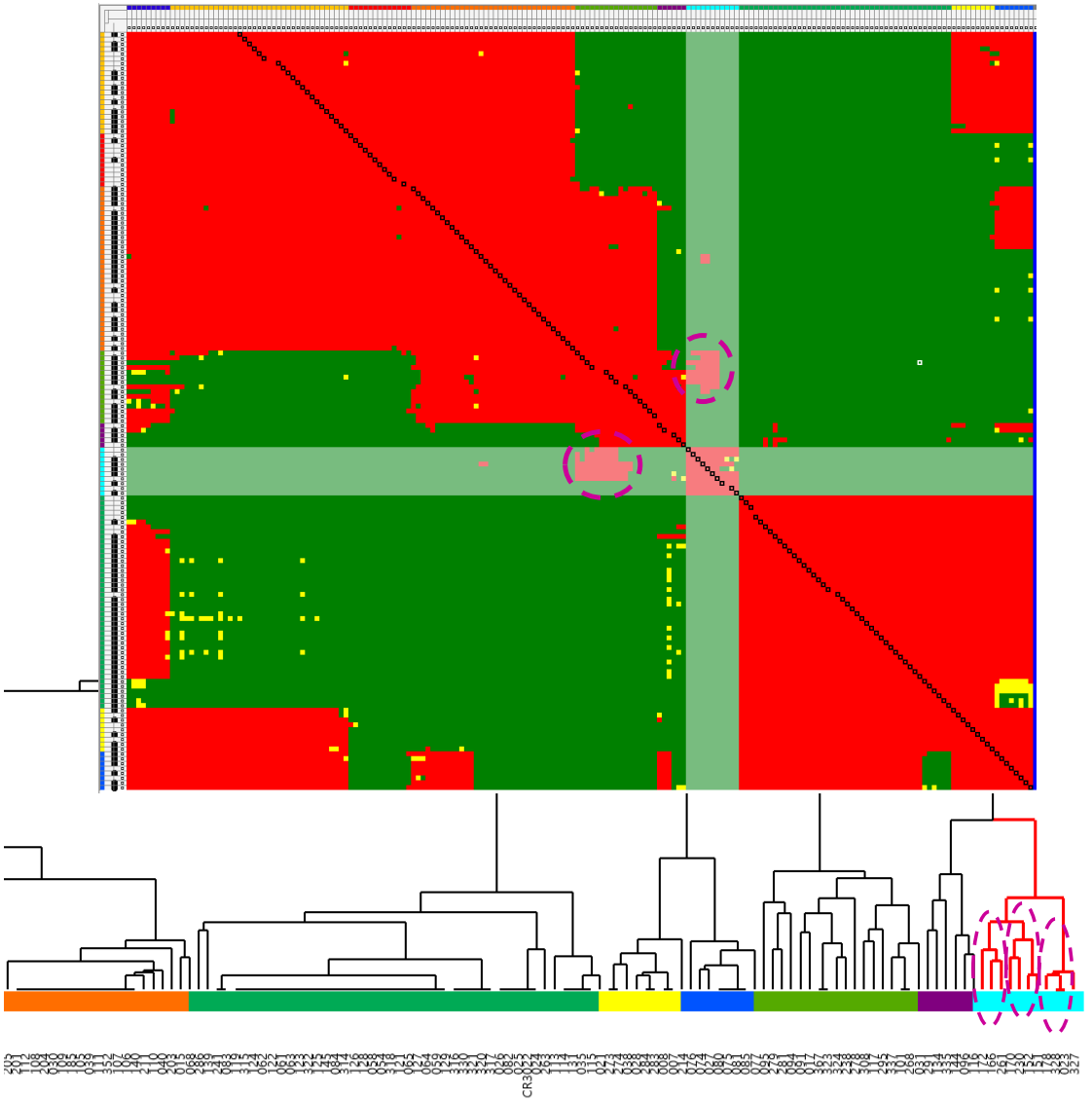


Community 4

- Competitive with Com 3 above, but differentiated by sandwich with the yellow and light blue community (Com 8 and 9)

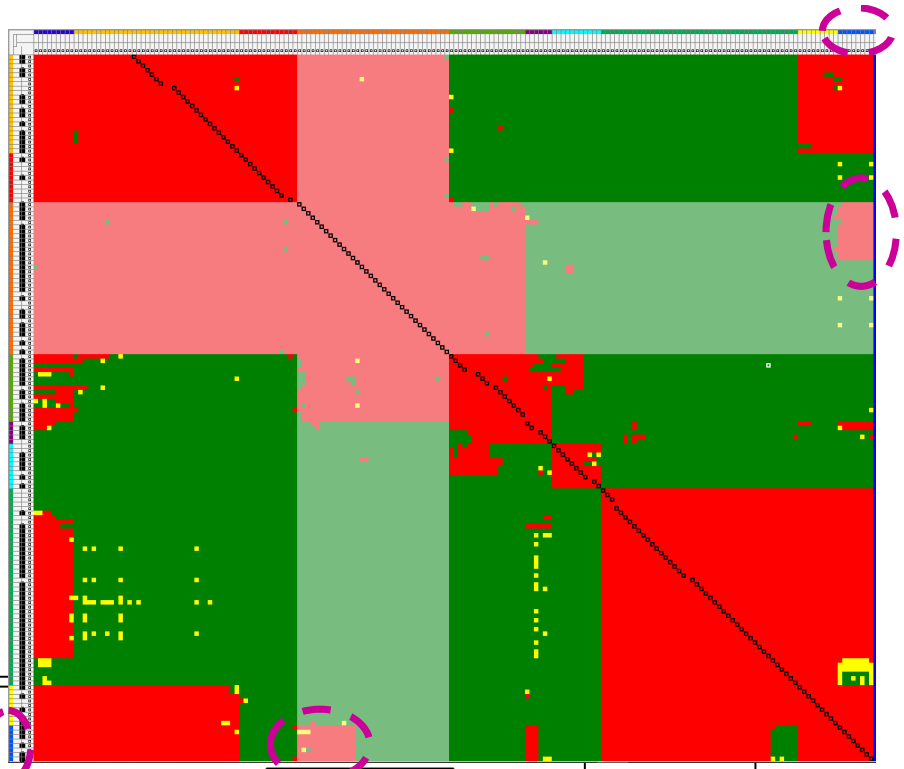
Community 5, A well differentiated cluster

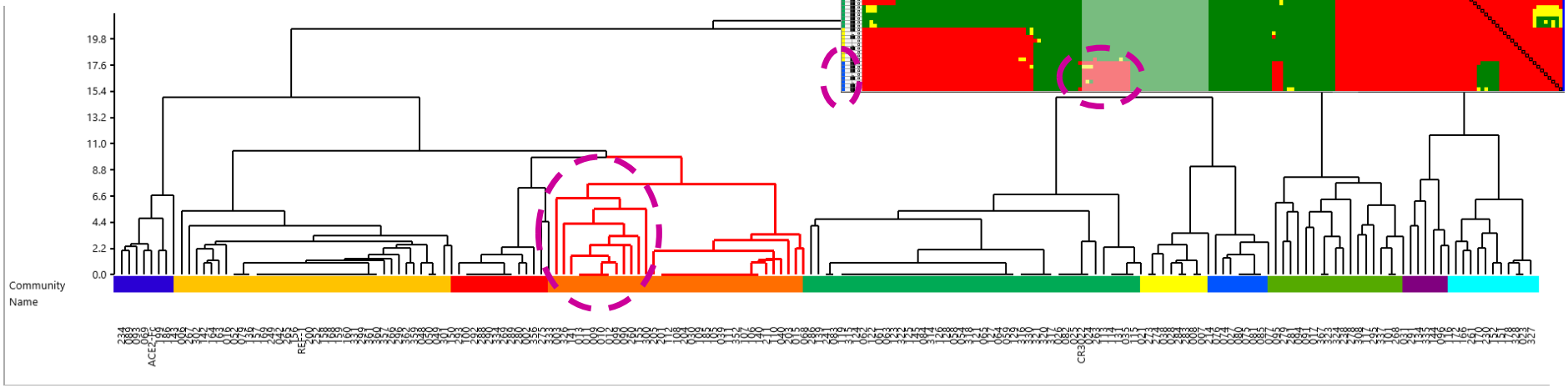
- This cluster is important as it is largely independent on the other clusters, with some small points of overlap with clones in the light green community
- These shared differences among some of the clones should likely be captured and reported (5A, 5B, 5C)
- The cluster does not fully violate the descriptive feature as it is a partial exception from one community



Community 6

- This community is technically over-clustered based on the “Rules for Good Communities”
  - It has a subset of clones which compete with all members of community 8 (light blue)
- Com 6 clones share many relationships and clearly overlap, but there is at least two populations with shifted epitopes within the community
  - These should be reported as sub-clusters for accuracy and funnel purposes (Com 6A, 6B)
  - Lowering the cutoff to support this separation splits the green community into 3 pieces





Takeaways

- The LSA facilitates large epitope binning experiments up to 384x384
- The array allows for inclusion of additional tests with no extra analyte sample and minimal effort
- Carterra's Epitope software allows display of orthogonal data in the heat map and network plots
- Assay optimization is essential
- Effective data curation simplifies the analysis and makes results more interpretable
- Community clustering is powerful and should be applied in a conscientious manner following some commonsense rules