Epitope binning analysis for further differentiation of therapeutic antibodies and diagnostic reagents

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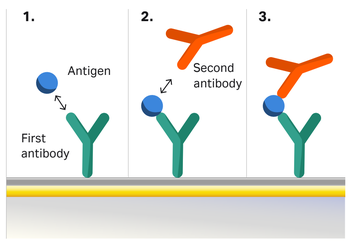
Background

Our intention with this study was to establish a well-characterized and easily accessible model system to be used for epitope binning assays using Biacore™ surface plasmon resonance (SPR) system. Our criteria for the model system were that a monovalent antigen and at least two antibodies should bind to the antigen simultaneously. The system we evaluated was epidermal growth factor receptor (EGFR) and three therapeutic antibodies targeting the extracellular domain III of EGFR. The antibodies were panitumumab (Vectibix®), cetuximab (Erbitux®), and matuzumab (Carbosynth). According to the previously published X-ray crystal structure, the Fab fragment of matuzumab (Fab72000) interacts with an epitope adjacent to the cetuximab epitope and should thus be able to form a sandwich complex with at least cetuximab. Panitumumab and cetuximab, on the other hand, recognize the same epitope and should thus block each other. This poster summarizes the results from epitope binning using sandwich and premix assay setups and our interpretation of the findings.

Epidermal growth factor receptor (EGFR)

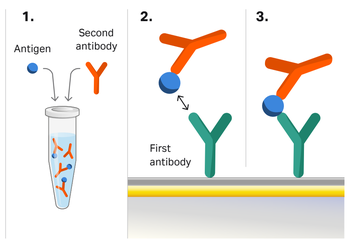
EGFR is a transmembrane protein and a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/neu (ErbB-2), Her 3 (ErbB-3), and Her 4 (ErbB-4). Mutations affecting EGFR expression or activity could result in different cancer types. Anti-EGFR monoclonal antibodies for therapeutic use bind to the extracellular domain of EGFR and block ligand-induced EGFR tyrosine kinase activation. In this study, we used the extracellular domain (Met 1-Lys 625) of human EGFR (Thermo Scientific™).

Sandwich assay



- In the sandwich assay, the first antibody is captured or covalently coupled to the sensor surface.
- The antigen is then injected over the first antibody followed by injection of the second antibody.

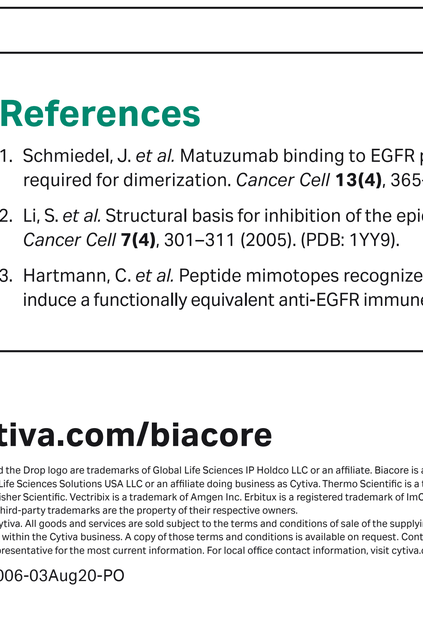
Premix assay



- In common with the the sandwich assay, the first antibody is captured or covalently coupled to the sensor surface in the premix assay.
- Prior to injection, the antigen and the second antibody are mixed outside the instrument.

Fab:EGFR complex

X-ray crystallographic data of the Fab fragments of cetuximab (1YY9) and matuzumab (3C09) in complex with the EGFR ectodomain III (1, 2) show non-overlapping epitopes for cetuximab and matuzumab as shown in the figure below. Although the epitopes are different, they are in very close proximity to each other.



Overlay of the X-ray structure 1YY9 and 3C09
Image of the Fab:EGFR complexes when superimposed via their EGFR domain III (green and orange). Fab of cetuximab (blue) interacts with EGFR (green). Fab of matuzumab (gray) interacts with EGFR (orange). Interacting residues in both EGFR domains are highlighted.

References

1. Schmiedel, J. *et al.* Matuzumab binding to EGFR prevents the conformational rearrangement required for dimerization. *Cancer Cell* **13(4)**, 365–373 (2008). (PDB: 3C09).
2. Li, S. *et al.* Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell* **7(4)**, 301–311 (2005). (PDB: 1YY9).
3. Hartmann, C. *et al.* Peptide mimotopes recognized by antibodies cetuximab and matuzumab induce a functionally equivalent anti-EGFR immune response. *Oncogene* **29**, 4517–4527 (2010).

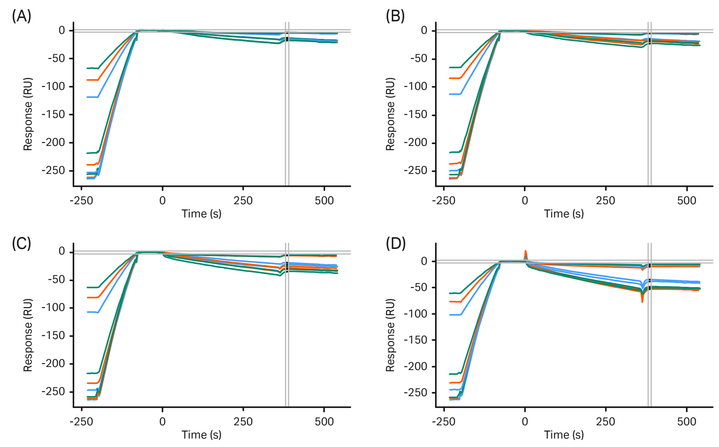
Epitope binning EGFR (Sandwich)

Experimental conditions

- Amine coupling of panitumumab (2549 RU), cetuximab (3456 RU), and matuzumab (3891 RU) to Sensor Chip CM5
- Assay buffer: HBS-EP+
- Analyte concentration: EGFR 40 nM
- Second antibody concentration: 4, 40, 400, and 4000 nM
- Flow rate: 30 µL/min
- Regeneration: 2 × 60 s, IgG Elution buffer pH 2.0 (Pierce)

Instrumentation
Biacore 8K

Results
No binding was observed at any of the concentrations tested. However, a concentration-dependent decrease in response, which may indicate a displacement, was observed when matuzumab and cetuximab were used as the first antibody and cetuximab was used as the second antibody. This was not observed for panitumumab.



Sandwich assay sensorgrams: second antibody injected at different concentrations. (A) 4 nM, (B) 40 nM, (C) 400 nM, (D) 4000 nM.

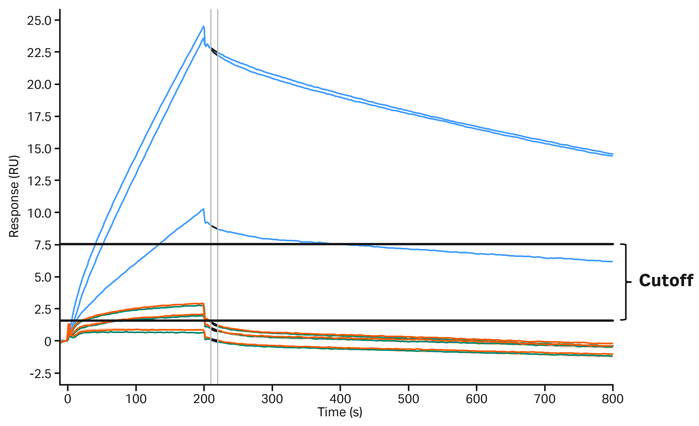
Epitope binning EGFR (Premix)

Experimental conditions

- First antibody immobilization: amine coupling of panitumumab (387 RU), cetuximab (439 RU), and matuzumab (505 RU) to Sensor Chip CM5
- Second antibody: panitumumab, cetuximab, and matuzumab
- Concentration premixed samples: EGFR 5 nM, second antibody 50 nM
- Assay buffer: HBS-EP+
- Flow rate: 30 µL/min
- Regeneration: 2 × 60 s, IgG Elution buffer pH 2.0 (Pierce)

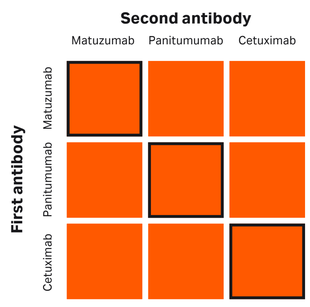
Instrumentation
Biacore 8K

Results
The results show that all three of the antibodies were blocking each other.

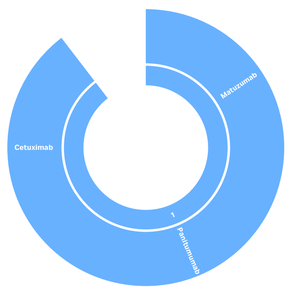


Premix assay sensorgrams: The blue curves, that is, above the upper cutoff, show the binding of EGFR alone. The other curves, that is, below the lower cutoff, show the antibody-antigen injections from the premix samples.

Results presented as heat map and bin chart



Heat map: A heatmap presents an overview of blocking, non-blocking, and uncertain antibody pairs. In this case, all antibodies are blocking each other, which is marked in red.



Bin chart: A bin chart presents the data in a different way. All three antibodies are blocking each other, which is illustrated here in blue.

If all three antibodies belonged to different bins, they would have been separated by "white background", that is, three different boxes as well as three different numbers and different colors.

Discussion

As shown by the Fab:EFGR X-ray data, the contact sites identified on EGFR with cetuximab and matuzumab do not overlap. However, the two antibodies may still contact overlapping surfaces, sterically preventing either one of them from binding to a receptor already occupied by the other. The figure does not represent a state possible "in real life", that is, with both antibodies bound, since it is an overlay of two different X-ray crystal structures and not a co-crystal of all relevant molecules. In addition to the published crystal structure, competition binding experiments with radiolabeled matuzumab and cetuximab on A431 cells have been performed, but no data from binding experiments that show simultaneous binding to the very same EGFR molecule in soluble EGFR were included (1). In our study, neither the sandwich or premix assay showed simultaneous binding of any of the antibodies tested.

The premixed assay was utilized to confirm the results from the sandwich assay. A previous study showed that two peptides, selected from random libraries by biopanning, both recognized matuzumab and cetuximab (3). These two peptides induced production of antibodies in rabbits that cross-reacted with human EGFR. The anti-peptide antibodies shared functional characteristics with cetuximab and matuzumab. We suggest that the binding epitopes of panitumumab, matuzumab, and cetuximab actually overlap in solution and thus block the binding of one another.

Conclusions

- Combination of data from different X-ray structures may not represent the "real-life" scenario; binding epitopes of panitumumab, matuzumab, and cetuximab may actually overlap in solution and thus block the binding of one another.
- The model system did not fulfill our criteria as all antibodies recognized the same epitope or exerted steric hindrance to block each other.
- Ability to measure binding at low immobilization levels and low sample concentrations requires a sensitive instrument —in this case, Biacore 8K—to distinguish real binding from artefact/bulk responses. These responses may occur when working with high surface and sample concentrations.

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