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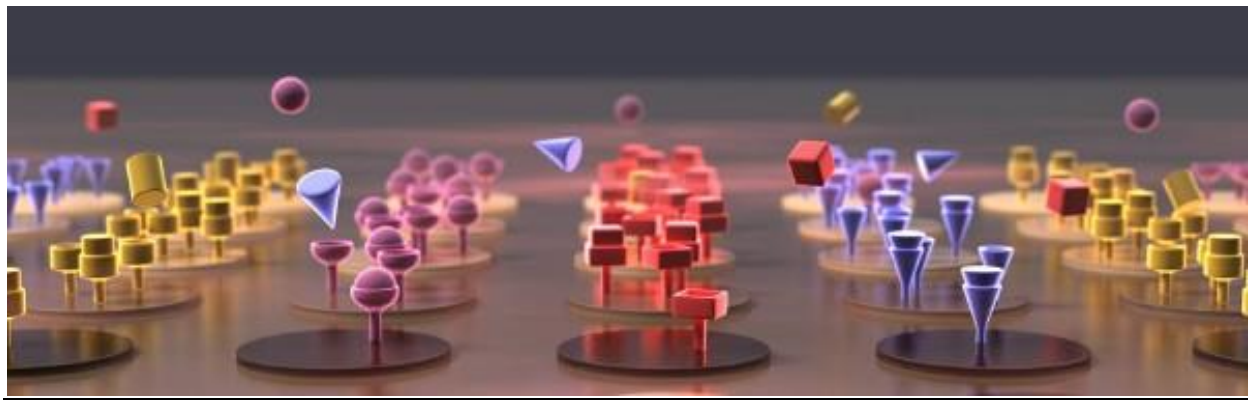

IBIS White paper

Epitope mapping at the amino acid level
 # 7.140605

1. Abstract

In this white paper, a new method is presented for the antibody epitope discovery at the amino acid level. Antigens contain many undetermined epitopes which all can be a target for antibodies. If the antigen amino acid sequence is known, linear epitopes can be defined through epitope mapping. Here we studied a total of 40 different 12-mer peptides, all biotinylated at the N-terminus, comprising amino acids 149 -201 of the RSV-G protein. This region of the protein contains the epitopes of two previously described antibodies (3D3 and 131-2G). The resultant 12-mer overlapping peptides were then used to create arrays on a streptavidin-coated (3D) SensEye® sensor which is inserted in the IBIS MX96 imager. The arrayed peptides are then exposed to the RSV-G protein specific antibodies 3D3, 131-2G and antibody AIMM-1 of AIMM therapeutics [1]. Each antibody only binds a specific group of peptides, from which the antibody epitopes can be defined. 3D3 and 131-2G bind a very similar epitope, with 131-2G requiring more amino acid contacts to bind. The unique AIMM-1 antibody bound to a new epitope, which is not recognized by 3D3 and 131-2G. The experiments were conducted on the IBIS MX96 imager in the labs of AIMM Therapeutics, Amsterdam, The Netherlands.

2. Introduction

In total 40 different 12-mer peptides, all biotinylated at the N-terminus, comprising amino acids 149 – 201 of the RSV-G protein, were coupled to a streptavidin-coated SPR sensor. After coupling, the chip was exposed to RSV-G binding antibodies. Based on the antibody response, an epitope map of each RSV-G binding antibody can be constructed, defining the minimal or core epitope (residues essential for binding) and the complete epitope of the antigen to the antibodies (core epitope + adjacent residues that are not essential for binding, but enhance the response).

3. Materials and methods

Biotinylated peptides were synthesized with amino acids according to table 1. The backbone of the antigen is the following linear sequence of 52 amino acids:

KQRQNKPPSKPNNDFHFEVFNFVPCSICSNNPTCWAICKRIPNKKPGKKTT

To couple the biotinylated peptides a streptavidin-coupled G-type chip (#1-08-04-008) (Ssens bv) was used. Now, the biotinylated peptides can be spotted to the recently developed G-STREP SensEye® sensorchip (# 1-09-04-008) (figure 1).

Figure 1. *Disposable SensEye® biosensor*

The use of the SensEye® biosensor is much more robust since it has been developed to avoid the use of refractive index matching oils and thereby speed, accuracy and resolution are improved.

Prior to immobilization of the peptides, the chip was washed with PBST (2 x 15 min at 10 µl/sec). To monitor the activity of streptavidin, a biotinylated mouse antibody was coupled to the chip as a control. Streptavidin-coupled mouse antibodies were detected using a goat-anti-mouse antibody. To monitor the activity and concentration of injected human antibodies, biotinylated anti-human IgG is spotted to the chip. Peptides and antibodies were diluted into PBST and spotting on the chip for 40 min, using a continuous flow microspotter (CFM) instrument. The CFM applies "flow cells" to homogeneously deposit biomolecules, in this case biotinylated peptides (12-mers), onto the biosensor surface. Unlike other systems, which deposit droplets to create non-homogenous features on a surface, the CFM 'circulates' the peptides back-and-forth over the surface, until complete binding saturation occurs wherever the peptide sample is in contact with the sensor surface.

After coupling the biotinylated peptides plus controls, the CFM-arrayed sensor was inserted in the IBIS MX96, and concentration series of antibodies, followed by the complete RSV-G antigen, were then sequentially injected and passed over the complete sensor. RSV-G antigen is injected to verify the activity of the injected human antibodies (the human antibodies are captured by the spots coated with biotinylated anti-human IgG). After interaction with the antibodies the array was regenerated (cleaned) using an acid step to remove the antibody and to prepare the sensor for the subsequent sample. In this experiment, antibodies 131-2G (mouse anti-RSV G, epitope: HFEVF [2]), 3D3 (human anti-RSV G, epitope: FHFEVFNF [3]), and antibody AIMM-1 (human anti-RSV G, epitope unknown) were used to validate our epitope mapping method.

1. KQRQNKPPSKPN	11. PNND**FHFEVFNF**	21. NFVPCSICSNNP	31. NPTCWAICKRIP
2. QRQNKPPSKPNN	12. NND**FHFEVFNF**V	22. FVPCSICSNNPT	32. PTCWAICKRIPN
3. RQNKPPSKPNND	13. ND**FHFEVFNF**VP	23. VPCSICSNNPTC	33. TCWAICK**RIPNK**
4. QNKPPSKPNNDF	14. D**FHFEVFNF**VPC	24. PCSICSNNPTCW	34. CWAICK**RIPNK**K
5. NKPPSKPNNDFH	15. **FHFEVFNF**VPCS	25. CSICSNNPTCWA	35. WAICK**RIPNK**KP
6. KPPSKPNNDFHF	16. **HFEVF**NFVPCSI	26. SICSNNPTCWAI	36. AICK**RIPNK**KPG
7. PPSKPNNDFHFE	17. FEVFNFVPCSIC	27. ICSNNPTCWAIC	37. ICK**RIPNK**KPGK
8. PSKPNNDFHFEV	18. EVFNFVPCSICS	28. CSNNPTCWAICK	38. CK**RIPNK**KPGKK
9. SKPNNDF**HFEVF**	19. VFNFVPCSICSN	29. SNNPTCWAICKR	39. K**RIPNK**KPGKKT
10. KPNNDF**HFEVF**N	20. FNFVPCSICSNN	30. NNPTCWAICKRI	40.**RIPNK**KPGKKTT

Table 1: Peptide array used for epitope mapping

Antibody epitopes shown in blue (131-2G), red (3D3, non-overlapping with 131-2G) and green (AIMM-1)

4. Results and discussion

Using the selection/de-selection feature of the IBIS-SPRintX software, samples or region of interests (ROI) can be independently selected for easy analysis of the binding of the antibodies to the synthesized peptides (figure 2).

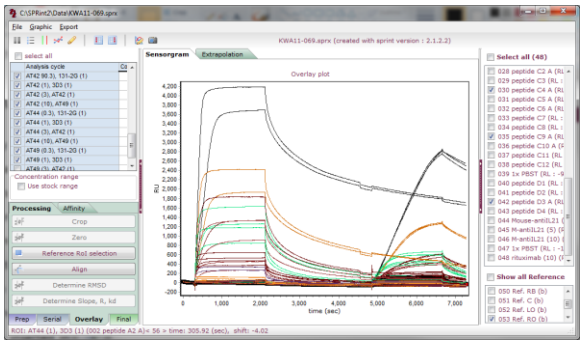

Figure 2. *Screenshot of the epitope mapping analysis from various injections of antibodies to the 12-mer peptides. Here typical specific binding to only a few spots occurred indicating the specificity of the antibody to at least the epitope core of RSV-G antigen.*

The interaction time has been set half an hour in order to measure close to equilibrium conditions at lower concentrations. Longer interaction times even up to 4 hours can be applied for high sensitivity and close to equilibrium measurements.

Figure 3 shows a sensorgram of the responses of the 3D3 antibody to the spots. The essential epitope core can be identified which consists of an 8 amino acid sequence FHFEVFNF.

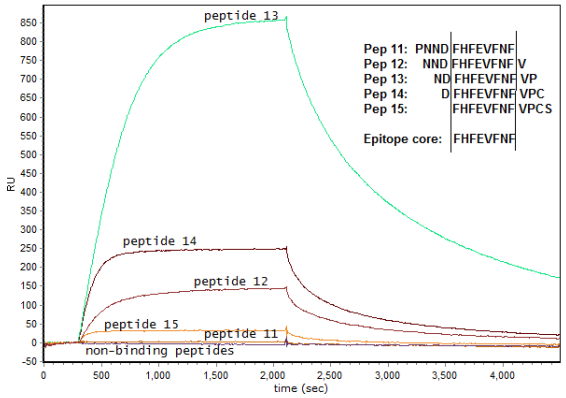

Figure 3. *Sensorgram of the 3D3 analyte interaction injected at 1 µg/ml to spots peptide 11 – peptide 15. The core of the epitope with the essential amino acids for binding of 3D3 antibody is FHFEVFNF After each antibody injection, the sensor was regenerated using glycine HCl pH 2.0.*

The two amino acids adjacent to the epitope core in peptide 13 contribute to an enhanced binding response. Because only a 12-mer peptide is used more amino acids may contribute to enhance the binding further.

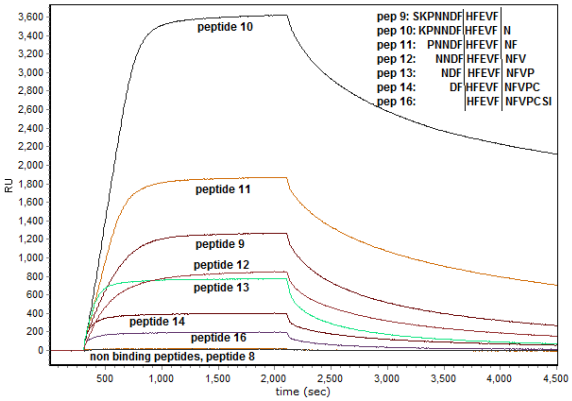

Figure 4*. Sensorgram of the 131-2G analyte interaction injected at 1 µg/ml to spots peptide 8 – peptide 16.*

Figure 4 shows the sensorgram of antibody 131-2G interacting with peptides 9-16. The antibody 131-2G binds peptides 9 to 16. This means that the core of the epitope consists of only five aminoacids: **HFEVF;** the six amino acids directly N-terminal to the core epitope of 131-2G enhance binding but are not essential. The core epitope of 131-2G is smaller than the *F***HFEVF***NF* epitope core of 3D3. Generally, the more peptides an injected antibody binds, the smaller its core epitope.

The third antibody, AIMM-1, recognizes an entirely different epitope, which lies ± 20 amino acids C-terminal to the epitopes of 3D3 and 131-2G (figure 5). AIMM-1 has the core epitope **RIPNK**; the amino acids *KPG*, adjacent to C-terminus of the core epitope enhance binding of AIMM-1. Together with the core epitope **RIPNK**, these form the complete epitope **RIPNK***KPG*.

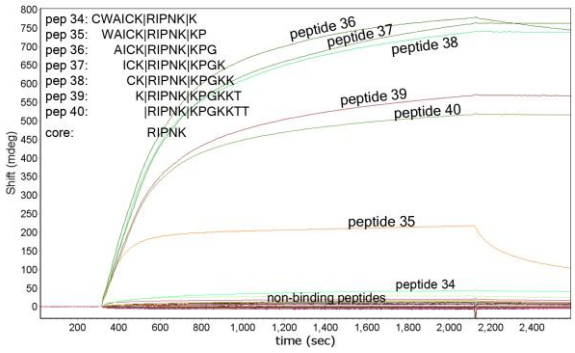

Figure 5: *Sensorgram of the AIMM-1 analyte interaction injected at 1 µg/ml to spots peptide 34 – peptide 40.*

An epitope map was constructed from the SPR results, showing which amino acids form the epitope of each antibody using the information gathered by the IBIS MX-96. In this map, the logarithmic maximal responses obtained from the sensorgrams (figure 6) are plotted as a function of the peptide number.

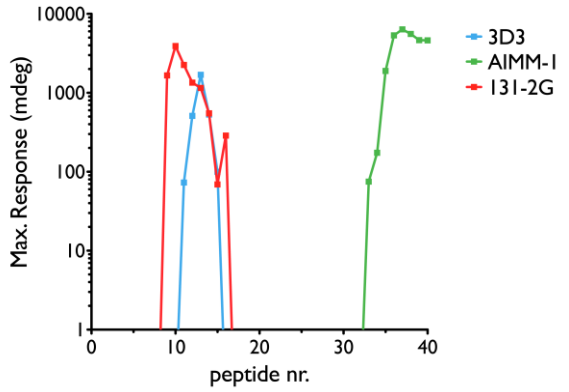

Figure 6. *Maximum responses 3D3, 131-2G and AIMM-1 as a function of the peptide number.*

5. Conclusions

The peptide scan array multiplex approach detects the linear epitope of an antigen to the interaction of a specific antibody at the amino acid level. Although many of the alternative epitope mapping tests are ELISA-based the reliability and accuracy obtained with the IBIS MX96 is unrevealed. Spotting of biotinylated peptides to streptavidin sensors is convenient and easy and the injection of samples is automated. One amino acid more or less changes the binding and affinity properties of the antibody dramatically. As shown here the two antibodies 3D3 and 131-2G compete for the same HFEVF epitope core of the RSV-G antigen. 3D3 has a larger epitope core and therefore binds less peptides that contain the essential sequence of HFEVF. Only peptide 11 till 15 contain this sequence FHFEVFNF that is essential for 3D3-binding. The AIMM-1 antibody appeared to have a different epitope, present in peptides 32 - 40. The AIMM-1 target seqeunce, RIPNKKPG, does not overlap with the core epitope of 3D3 and 131-2G. Thus, we have identified a new epitope in the RSV-G protein, which is the target of the AIMM-1 antibody.Using our IBIS-SPR epitope mapping, we can also distinguised between the core epitope and the complete epitope. The core epitope contains the residues that are essential for antibody binding. We have shown that some adjacent amino acids enhance the binding response but are not essential for binding. Together with the core epitope, these enhancing residues form the complete epitope.

For example, we have shown that the core epitope core of 131-2G is only 5 amino acids (HVEVF). The KPNNDF amino acids directly N-terminal to the core HFEVF dramatically enhance binding of this antibody; suggesting that the complete epitope of 131-2G is KPNNDFHVEVF. So used, the IBIS-MX96 facilitates epitope mapping at amino acid level, and allows the definition of essential residues within antibody epitopes.

6. Summary

Important features and benefits of the IBIS MX96 instrument are demonstrated in this white paper. It opens up new possibilities for researching the nature of epitopes of antigens at the amino acid level. We have validated the (previously described) epitopes of antibodies 3D3 and 131-2G, in tandem finding which residues within their epitopes are essential for binding. In the same experiment, we have mapped the epitope of antibody AIMM-1, revealing that this antibody binds a new, unique, epitope in the RSV-G protein.

The results presented in this white paper are supportive to the fact that the IBIS MX96 system is the versatile, robust multiplex workhorse of choice to expand the discovery horizon of epitope mapping of antibodies. The unique design of this instrument allows high-throughput epitope mapping at single amino acid resolution.

7. References

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