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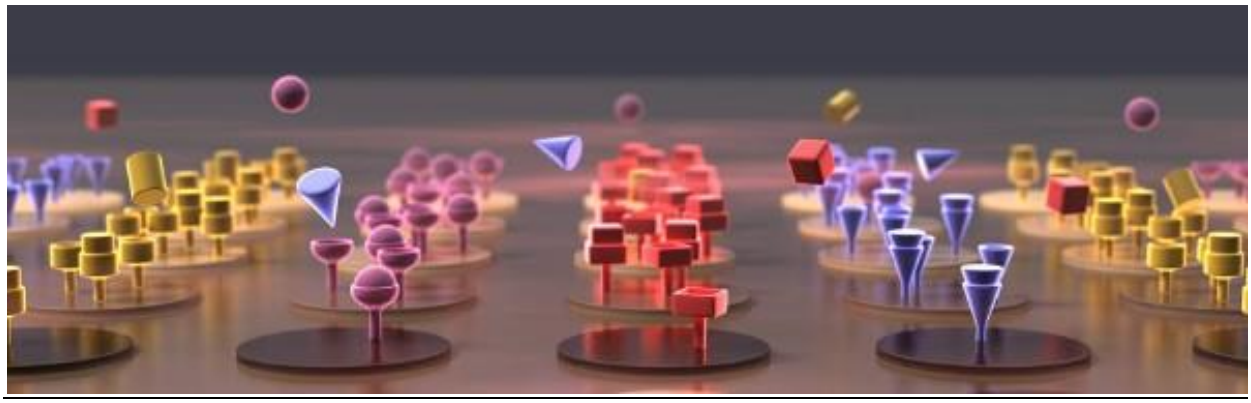

IBIS White paper

Combined ranking and binning

#12.141209

1. Abstract

In this white paper #12, the results are described of a combined kinetic ranking and binning study performed at UCB Pharma (Slough, UK). The ranking and binning was for 9 different antibodies using a single sensor, but up to 96 antibodies could be tested using a similar procedure as described in this WP#12. Each antibody was spotted 5 times in a two-fold ligand density series for not only finding the affinity towards its target (Protein X) but also for identifying the bins (blockers and sandwichers, see also WP#6). Due to the various ligand densities that were spotted the K_D^{R0} method could be applied extrapolating the R_{max} (a measure of ligand density) to zero. Also, a recognized global analysis evaluation using Scrubber2 software was carried out in order to compare with known "Biacored" values at UCB. The average, global and K_D^{R0} values are given in this report for the clones directed toward Protein X. The experiment showed ranking results similar to previous tests as measured on different label free kinetic platforms at UCB.

The binning experiment showed a clear result of 3 bins and the best affinity antibody in these bins could be identified. Software for kinetic ranking and binning is available to analyze the data with enormous speed and accuracy. Hence the IBIS MX96 can become the high-performance work-horse for performing combined ranking and binning of antibodies to functional epitopes for finding the best therapeutic candidate in a limited period and at the lowest costs which is unrivalled in the market of high-end label-free interaction analysis equipment.

2. Introduction

A demonstration of IBIS equipment was organised at UCB Pharma, Slough, UK in July 2014. Two tests were originally planned. An affinity ranking test of 9 purified antibodies to a single antigen (anonymised "Protein X") followed by a binning experiment with the same 9 antibodies to address competing and non-competing antibodies to this "Protein X". All 9 antibodies were immobilized on an Easy2Spot (E2S) sensor and the "Protein X" acted as the analyte. To determine affinity parameters, the new affinity determination method was applied among the standard global analyses and will be described more extensively in the appendix of this WP#12. The enormous multiplexed data stream from the IBIS MX96 was processed in SPRintX 1.9 software and the binning tool software developed by Wasatch Microfluidics (producer of the Continuous Flow Microspotter). The selection of data in SPRintX can be exported to a Scrubber file using the export data tool (*.ibmx). Scrubber2 is a data analysis software package developed by BioLogic Software, Pty Ltd, Australia. In the appendices additional information is given for reporting a kinetic strategy which has been embedded in new SPRintX 1.9 software.

3. Materials and methods

3.1 Samples

Protein X was tested as the analyte at concentrations of 100 to 3.1 nM in a two-fold serial dilution. Using the CFM spotter 45 spots were loaded with 9 different antibodies to this target in a two-fold dilution series of five different concentrations starting at 100 nM. Sensors with these ligands were prepared by covalently coupling these antibodies to the sensor using the Easy2Spot sensor. Remaining spots were used for controls and blanks.

3.2 Sensor preparation

Surface Plasmon Resonance imaging analyses were performed using E2S SensEye® SPR sensors (Ssens BV, Enschede) followed by immobilizing the ligands using a Continuous Flow Microspotter (CFM, Wasatch Microfluidics (Salt Lake City, Utah, US). Ligands were immobilized in 50 mM NaAc buffer, pH=4.5 using the CFM. An array of 48 spots (including controls) was obtained. As already indicated it is described in several publications that the distribution range of affinities is affected by the ligand immobilization process. Therefore, a ligand density at 5 concentrations was applied in order to find a best estimate to an unaffected affinity value. Ligands and controls were immobilized for 30 minutes. Control spots contained human serum albumin (0.5 g/L) or were ''blank' using 50 mM NaAc buffer, pH 4.5.

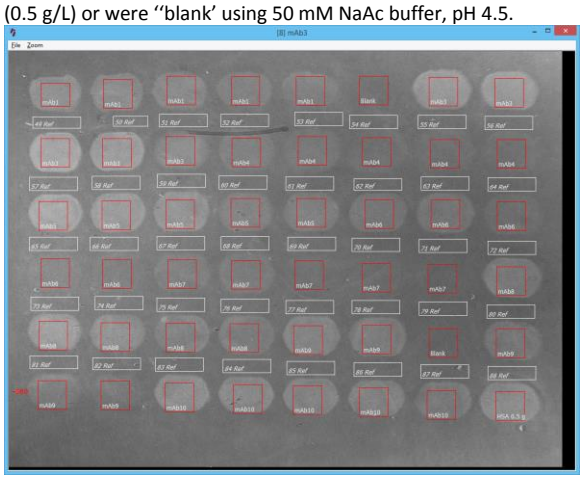

Figure 1. *Surface Plasmon Resonance image of the SensEye with spotted UCB ligands.*

3.3 Interaction measurements

After installing the SensEye® containing the array in the IBIS MX96 the remaining activated esters were deactivated for 7 minutes using 1 M Ethanolamine pH 8.5 and the regions of interest (ROI) were positioned on the ligand spots. Reference ROIs were positioned on the sensor regions where no ligand was spotted. Samples were exposed to all these spots so interactions were followed in 48-plex format. Also all reference signals in 40-plex format were detected to enable local referencing of the signal spots. The running buffer being used was PBS-Tween80.

3.4 Analyte analysis

All analytes were diluted in running buffer to minimize refractive index bulk shifts and injected over the ligand array in 6 concentrations in a two-fold dilution starting at 100 nM and ending at 3.1 nM. A continuous flow of running buffer was supplied to the sensor during the dissociation phase of the interaction in order to remove dissociating analyte. After dissociation, the sensor surface was regenerated with 10 mM Glycine HCl, pH 2.0 buffer for 60 seconds. The sensor surface was washed with running buffer between each injection. Subsequent to the analyte injections, samples of running buffer containing different glycerol concentrations were injected to obtain a calibration plot to correct for unequal sensitivity of ROI's to a bulk refractive index shift caused by various ligand densities and potential heterogeneities observed at the sensor surface.

4. Results and discussion

4.1 Processing of the data in SPRintX software

The in-house developed K_D^{R0} method as described in *Nature*[1] and *Analytical Biochemistry*[2] is employed to obtain the K_D^{R0}, k_d^{R0} and k_a^{R0}. Briefly, off-rate k_d^{R0} and affinity equilibrium constant K_D^{R0} are determined by exposing an array of spots on the sensor surface containing varying ligand densities to varying concentrations of analyte. By plotting the k_d and K_D against the measured responses, the k_d^{R0} and K_D^{R0}at a response of 0 can be determined by means of extrapolation. These values are considered the best approximation of a true 1:1 interaction. In theory when the ligand density is extremely low the actual "solution" single molecular analyte-ligand affinity constants (rate and dissociation equilibrium constants) can be determined. On-rate k_a^{R0} is then calculated by dividing k_d^{R0} by K_D^{R0}. (See appendix and the papers.)

The advantage of the method is that by using the array to create a ligand density series of a limited number of antibodies, the optimal ligand density for determining the affinity parameters is always present. The disadvantage is that a large panel (e.g. 96 antibodies at once) cannot be tested with the K_D^{R0} method simultaneously. However, global ranking per spot can then be applied. In this study a total of 45 spots was applied for 9 antibodies (five different densities each). Because for the K_D^{R0} method the local affinities for each analyte injection over each spot gives us a local value of k_d, k_a and K_D, a ranking value of these local affinities can be averaged (under specific restrictions excluding outliers) and the spots (with antibodies) can be ranked in terms of the affinity parameters. The SPRintX processed data can be CRAZE'd (Calibrating, Referencing, Aligning, Zeroing and Exporting) in order to create an *.ibmx file which can be imported in the binning tool. Also, a processed *.ibmx file can be exported to Scrubber2 to perform world-wide recognized global affinity calculations.

4.2 Affinity constant determination

As mentioned, the K_D^{R0} method was used in SprintX to determine the affinities to the several ligands. The method follows the strategy of the current global analysis methods and applies the ligand density series as measured in the IBIS MX96. Data obtained for all injections is pooled and will be used for determining the affinity constants by applying the K_D^{R0} method.

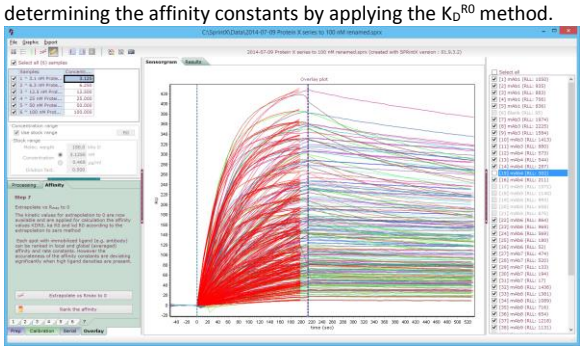

Figure 2. *Overlay plot of the biomolecular interaction responses with 6 analyte injections over all ligand spots (see also figure 1) and fitting the association phase. A broad range of affinities can be observed. The combined data of these interactions will be used to determine the local rate and affinity constants. Fortunately, the capacity of the IBIS MX96 is much higher, only 6 analyte injections were relevant for the test. Best for high quality data is to start at a low concentration and increase the concentration of the analyte in a three-fold series. (So-called kinetic titration)*

IBIS SprintX software includes a wizard that allows easy determination of K_D^{R0} values using overlay plots of a massive number of sensorgrams. The wizard first references, zeroes and aligns the sensorgrams, so using the automated CRAZE function in the software. Next, the off-rate k_d will be determined by setting rulers at the appropriate location on the dissociation phase. The wizard will automatically deselect the interactions whose off-rates could not be determined. Also linear responses without curvature should be deselected since these generate too high theoretical R_{max} values. This can be easily obtained by using the SPRintX analysis software.

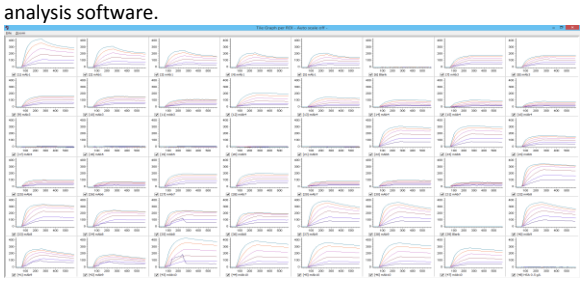

Figure 3. *Tile plot of spots with 6 analyte injections in overlay. A wealth of screening information can be obtained in a single run of about 2 hours.*

Although this experiment is limited to only 9 different mAb spots, 48 or 96 spots (double print) can be tested in a single run. In this case 96 mAb's can be measured in about 2 hours for consistent affinity values. Preferably 8 injections should be optimally performed to cover the range of extreme low to high affinities of the Ab to the analyte. In this case in a reasonable time frame more than 700 local off –rates and on-rates are automatically determined in two hours run-time only. The spots will be ranked in terms of global off-rate and equilibrium constants using an 1:1 interaction binding model which is the golden standard for ranking the affinity. Last but not least, only 100 µl of (precious?) antigen at low concentration (~20 nM) is injected for all these interactions simultaneously.

4.3 Binning of Protein X

A binning experiment was performed on the same SensEye sensor (figure 1) as the kinetic ranking experiment of the antibodies towards Protein X. In this way the affinity data could be directly combined with the binning data. The strategy is to find the best affinity antibody in combination with an unique bin. The IBIS MX96 can perform this antigen (Protein X) kinetic evaluation and a subsequent binning experiment in 4 hrs. In this way both the binning and affinity ranking data from only one spotted SensEye can be combined.

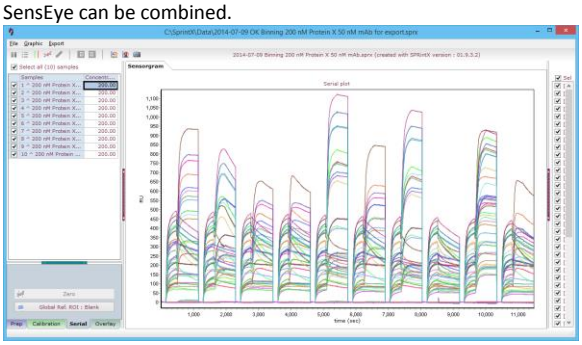

Figure 4. *Serial plot of the binning run. Clearly blocking and sandwiching antibodies can be observed. The 8th injection is a buffer injection after the antigen loading. Great reproducibility was obtained.*

The preprocessed (CRAZEd) data was exported to the binning tool. Below in figure 10 the prioritized sorted data is shown.

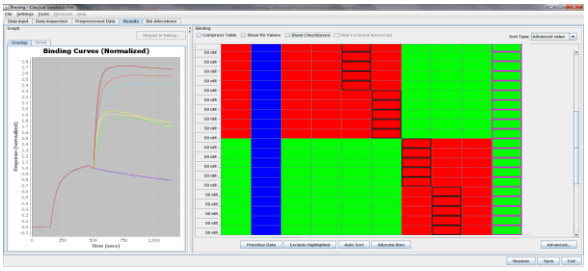

Figure 5. *In the graph shown on the left each individual sensorgram can be inspected. The organization of columns and rows is in such a way that similar antibodies are grouped. Blue is the buffer. On the right antibody mAb5 showed up as an orphan. Inspection showed that the mAb5 spot was dead. See figure 9.*

This resulted in the following allocated bins. The square mAb5 is unidirectional but is in a separate bin from the others. The reason that antibody mAb5 is not immobilized adequately is not known but if mAb5 is e.g. highly glycosylated it is less effective to couple using EDC-NHS coupling chemistry.

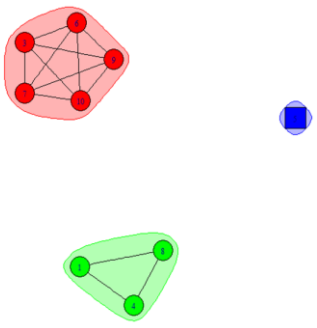

Figure 6.*. Allocation of the bins. Three clearly independent bins were found.*

The two best affinity antibodies in Bin 1 are antibodies mAb7 and mAb3. In Bin 2 antibody mAb8 showed the highest affinity. The third bin contained antibody "mAb5" of which the affinity could not be determined because of "dead" spots. However "mAb5" turned out to be a good sandwicher (as analyte).

Bin	mAb name	K_D
Bin 1	mAb7	0.15 nM
Bin 1	mAb3	0.2 nM
Bin 1	mAb6	0.8 nM
Bin 1	mAb10	3 nM
Bin 1	mAb9	4 nM
Bin 2	mAb8	0.9 nM
Bin 2	mAb4	3.5 nM
Bin 2	mAb1	5 nM
Bin 3	mAb5	ND

5.0 Conclusions

The combined ranking and binning study revealed 3 bins. The "best" highest affinity antibody in the first bin is "mAb7" close to "mAb3" with ranked K_D^{R0} of 0.15 nM and 0.2 nM respectively. For bin 2 the "mAb8" with K_D^{R0} = 0.9 nM was best among three. The third bin only contained "mAb5" . The analysis was based on K_D^{R0} extrapolation and local average affinity data for the covalently immobilized antibody.

In order to analyse sensorgrams for binning, the data was first processed using SPRintX software. All data is calibrated, referenced, aligned and zeroed (CRAZ) followed by K_D^{R0} extrapolation (E) to determine the K_D^{R0}, k_d^{R0} and k_a^{R0} affinity constants for all interactions. Remarkably even crude non-affinity purified supernatants that were directly covalently coupled using the CFM showed consistent data.

Appendix

Introduction to k_d^{R0} and K_D^{R0} as the best estimate rate- and affinity constants.

Since the K_D is used for the determination of the effective dosage levels of biomolecules that are applied as drugs, the determined K_D should reflect the K_D in solution. However, the constants that are derived from current, immobilized ligand based assays are affected by the immobilized state of the ligand. This causes the thus determined, apparent constants to deviate from the true, 'solution' constants due to interfering effects that result from the immobilization of the ligand. These interfering effects include rebinding effects, mass transport limitation, non-specific binding, deviation from 1:1 model binding etc. The higher the ligand density, the more pronounced these interfering effects become and it is generally accepted that the ligand density should be applied just above the limit of detection of the biosensor instrument. However, even at low responses the interfering effects may occur substantially. The same holds for the analyte concentration; interfering effects will occur when multiple analyte molecules compete for interaction with a single immobilized ligand molecule.

In this white paper a so-called K_D^{R0} method for the determination of affinity constants has been applied in which the contribution of interfering effects is minimized or theoretically zeroed, so that the constants are a better estimate of the true constants of bio-molecular interactions in solution. This method is based on the extrapolation of the number of immobilized ligand and analyte molecules to zero, thus mimicking the interaction in which only one ligand and one analyte molecule are involved, enabling a true 1:1 binding model with no or minimal interfering effects. In theory, the method compensates:

1. Parallel binding processes including non-specific binding and/or cross reactivity, dimerization or formation of multi assembly complexes.
2. Rebinding of dissociating analyte molecules including avidity and steric hindrance effects.
3. Non 1:1 binding model interactions including multi-phasic behaviour and bridging by molecules having more than one paratope.
4. Mass transport limitation effects, e.g. analyte depletion of the layer close to the immobilized ligand surface.
5. Additional interfering effects that do not fit into one of these groups, including complex reactions, e.g induced fit or allosteric conformational change.

Recognized effects are ligand immobilization artefacts and heterogeneity of surface binding sites. The method will not compensate for this and the "escape" route is by capturing ligands followed by the target interaction. Data obtained from direct ligand immobilization with respect to captured ligand are sufficiently different and this definitely is a proof that immobilized ligand affinity constants are affected by the immobilization process.

As already indicated, the calculation of the "true" affinity equilibrium constant will become more reliable at lower densities, preferably at a 'density' of only a single immobilized ligand molecule acting as a free ligand. Then the contribution of the interfering effects will be zero and will not influence the rate- and affinity equilibrium constants anymore. It should be noted that immobilization artefacts and heterogeneity of surface binding sites should be prevented for instance by oriented capturing of the ligands by applying high affinity anti-ligand antibodies or using tag – anti-tag interactions.

The IBIS MX96 instrument applies a valve-less injection of samples. Patented "back-and-forth" flow-based fluidics enables unlimited interaction times using only 100 microliter sample volumes that can be recovered. The unsurpassed multiplexing capacity of this platform provides scope to interrogate multiple interactions, simultaneously, whilst offering multiple referencing possibilities. Combined with the uncompromised data quality and reliability, the IBIS MX96 is positioned as the most technically advanced system available for label-free array analysis of biomolecular interactions. Important features and benefits of the IBIS MX96 which differentiate this system from other commercially available offering will include the combination of:

- Ultra low analyte consumption of 100 microliters that can be fully recovered,
- Unlimited interaction times enabled by back and forth mixing
- SPR imaging allows real-time "visual"' assessment of array quality and analysis.
- Largest dynamic and linear range enables full compensation of "common mode" effects by referencing.
- Multiplexing with 48-plex array fabrication (CFM).
- Local (individual) referencing for 48-96 reaction spots and calibration of the ROI sensitivity reveals unsurpassed quality on multiplexed data.

It has been clearly shown that the IBIS MX96 System offers distinct quality advantages over traditional non-imaging SPR approaches. For screening purposes, the IBIS MX96 in combination with the CFM spotter shows uncompromised quality together with a giant leap in reduction of analysis time. The researcher will concentrate on the biomolecular interactions in relation to positive, negative, reference controls. The instrument has contributed to ground breaking research by well-known scientists. This versatile, flexible research instrumentation delivers great value and uncompromised quality!

References

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