PROTEIN INTERACTION ANALYSIS

ProteOn™ XPR36 System Application Guide

- High-sensitivity multiplex SPR technology
- Real-time, label-free kinetic, specificity, and affinity data
- Automated analysis of up to 36 biomolecular interactions in one experiment
- Simplified hybridoma screening and ranking, epitope mapping, kinetic analysis, and cross-reactivity determination

Antibody Characterization and Development Using the ProteOn XPR36 Protein Interaction Array System

High-Throughput XPR™ Technology

The ProteOn XPR36 protein interaction array system is a 6 x 6 multichannel surface plasmon resonance (SPR) platform suited to a variety of antibody research interests. It combines the many advantages of highly sensitive SPR technology with high-throughput capability. XPR data are acquired without fluorescent or radiochemical labels. Real-time kinetic, specificity, and affinity data may be used to accurately and rapidly characterize antibody-antigen interactions for antibody development and screening.

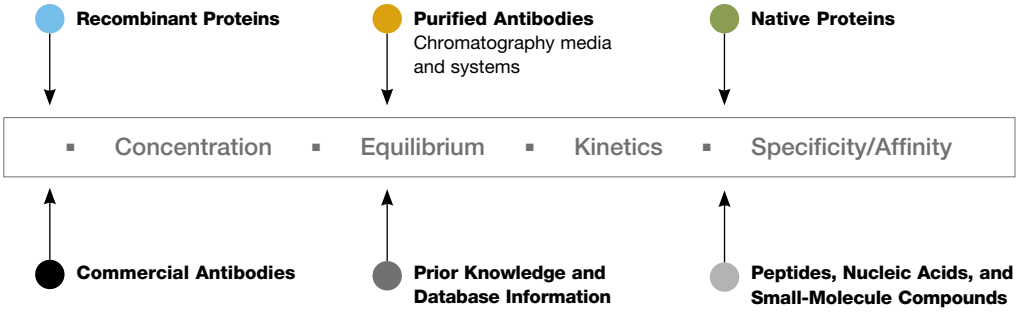
A powerful tool for life science research, the ProteOn XPR36 system provides automated, simultaneous analysis of up to 36 biomolecular interactions in a single experiment. Up to six antibodies may be simultaneously immobilized in parallel channels, and then assayed with panels of up to six antigens in orthogonal channels.

Following data acquisition, the immobilized antibody surfaces are regenerated, and a new assay is ready to run.

In this application guide, four antibody characterization and development protocols that demonstrate the versatility of the ProteOn XPR36 system are briefly described:

- Hybridoma screening and ranking
- Epitope mapping
- Kinetic analysis of antibody-antigen specificity
- Determination of cross-reactivity of antibodies used in multiplex assays

Two different methodologies are described, depending on whether the monoclonal antibodies were purified first. For unpurified samples, a capture antibody is immobilized to the surface of the chip before injection of the monoclonal. Purified monoclonal antibodies are immobilized directly on the chip surface.



BIO-RAD

Hybridoma Screening and Ranking

Hybridoma Screening

The objective of hybridoma screening is identification of hybridomas expressing high-affinity antigen-specific monoclonal antibodies. But the screening process itself may become overly time-consuming because conventional end-point assays (for example, ELISAs) often prove to be less than ideal for hybridoma screening purposes. For example, antibodies with similar affinities may display different off rates (dissociation of antigen from antibody). Screening hybridomas in real time on the basis of on and off rate in addition to affinity (end point) is a significant advantage of XPR technology. The additional advantage of 6 x 6 processing means hundreds of supernatant antibodies may be rapidly and accurately screened in just a few hours with nanomolar sensitivity.

In a typical XPR hybridoma screening experiment (Figures 1 and 2), purified capture antibodies are immobilized in six parallel channels using the amine coupling reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and *N*-hydroxy-sulfosuccinimide (sulfo-NHS). Unpurified supernatant antibodies for an antigen of interest (here, IL-9) are injected orthogonally. Next, the purified antigen of interest is injected in the same orientation as the original capture antibodies. XPR analysis yields 36 sensorgrams that detail the kinetics (affinity) of all 36 interactions in real time without the use of labels. Data are stored and analyzed using a variety of computational interaction models, typically 1:1 Langmuir binding. (For details, see Peterlinz and Georgiadis 1996.) After regeneration with dilute phosphoric acid, the sensor chip is ready for another 6 x 6 analysis of a second panel of hybridoma supernatant clones.

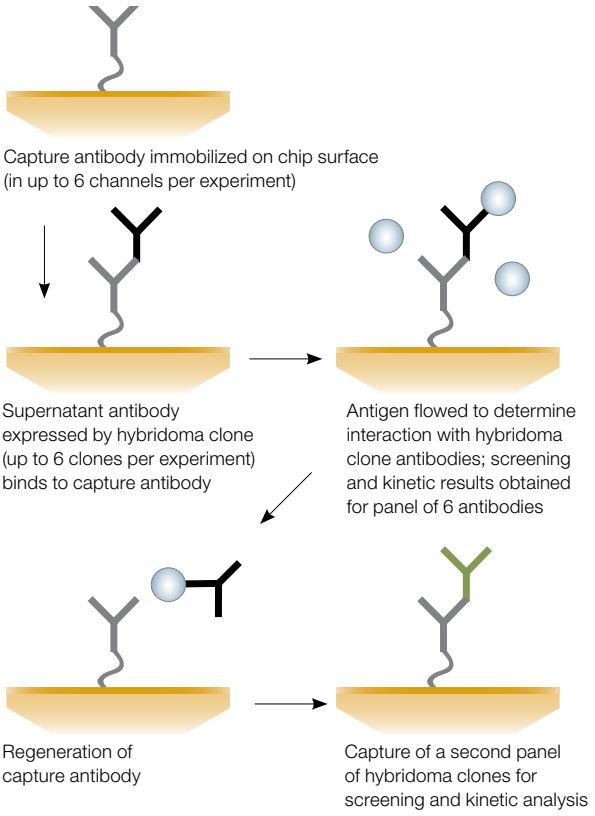


Fig. 1. Representative hybridoma screening workflow.

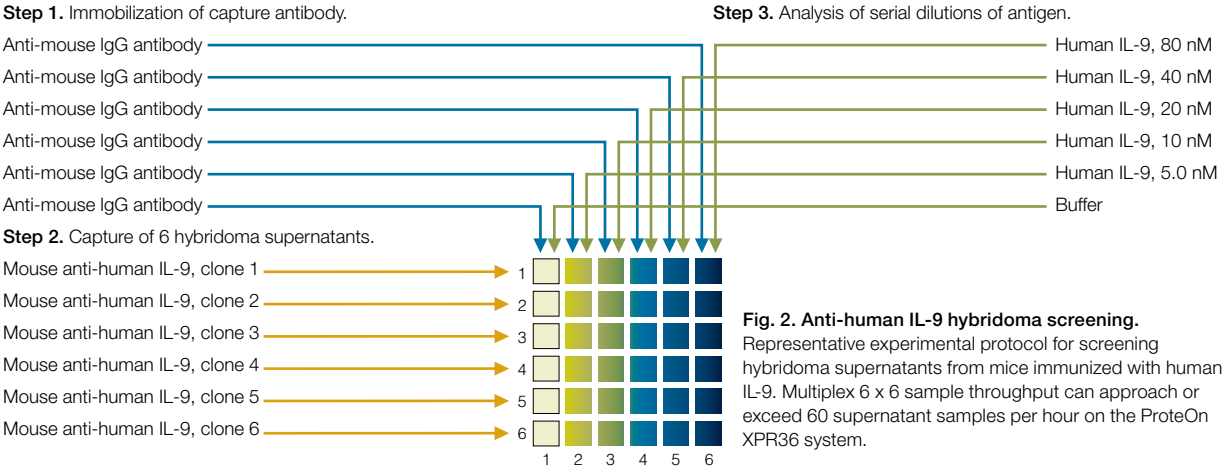


Fig. 2. Anti-human IL-9 hybridoma screening. Representative experimental protocol for screening hybridoma supernatants from mice immunized with human IL-9. Multiplex 6 x 6 sample throughput can approach or exceed 60 supernatant samples per hour on the ProteOn XPR36 system.

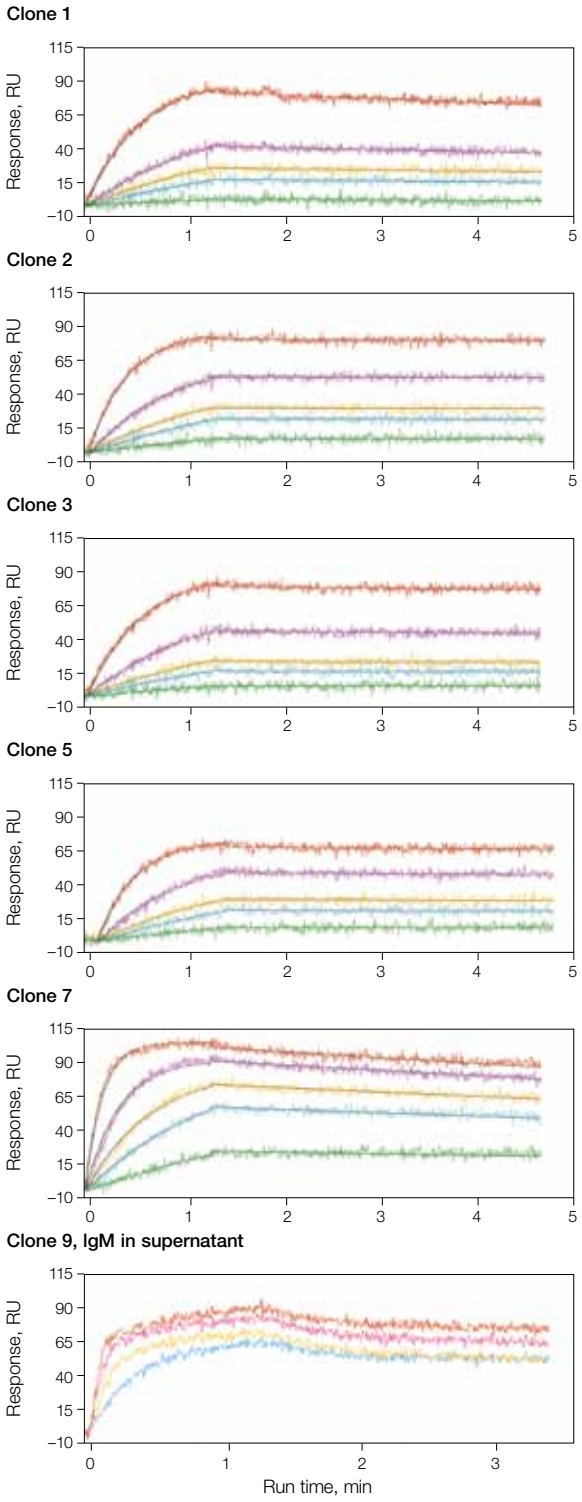


Fig. 3. Rapid screening of unpurified hybridoma supernatants to obtain ranking data. Shown are sensorgrams of six hybridoma clone supernatants, five exhibiting conventional 1:1 Langmuir binding, and a sixth displaying complex binding kinetics due to the presence of IgM in its supernatant. Black lines represent global fits of the sensorgrams to the 1:1 Langmuir interaction model. See Table 1 for the kinetic rate constants of these data.

Table 1. Kinetic analysis and ranking of anti-human IL-9 supernatants of selected clones. Ranking by highest to lowest affinity.

Clone	k_a, $M^{-1}sec^{-1}$	k_d, sec^{-1}	K_D, M
2	7.0×10^4	4.8×10^{-5}	6.9×10^{-10}
5	8.3×10^4	1.2×10^{-4}	1.4×10^{-9}
3	4.5×10^4	1.1×10^{-4}	2.4×10^{-9}
7	2.1×10^5	6.0×10^{-4}	2.9×10^{-9}
1	6.5×10^4	5.0×10^{-4}	7.7×10^{-9}
9	—	—	—

Hybridoma Ranking

Kinetic data can be used to rank antibodies expressed by hybridomas that are selected on the basis of earlier screening. Large numbers of hybridoma supernatants can be screened in successive cycles of analysis using the same sensor chip. To obtain the data in Figure 3 and Table 1, antibodies from 20 hybridoma supernatants were captured and their kinetic constants determined. Shown are results for five of the selected clones that produced antibodies with similarly high affinities (K_D <10 nM). Antibody from clone 2 had a slightly higher affinity (lower equilibrium dissociation constant, K_D value) and lower dissociation rate constant (k_d value) than the others, thus earning the highest ranking. The supernatant from clone 9 contained IgM antibodies, so the kinetics deviated from a simple 1:1 interaction model. (For details, see Bravman et al. 2007.)

Ranking on the basis of both affinity and rate of dissociation allows antibodies from the most promising hybridomas to be chosen for more detailed kinetic analysis. Because reliability of kinetic data increases with analysis of more purified antibodies over progressively defined concentration ranges, the advantages of hybridoma screening and ranking using the ProteOn XPR36 system are clear.

Epitope Mapping

One epitope mapping technique is the determination of whether two or more antibodies specific to the same antigen bind at different epitopes of the antigen. In pairwise epitope mapping, an antigen is assayed sequentially with two supernatant antibodies.
If significant binding occurs with the second antibody, the antibodies bind to different epitopes of the antigen. If not, the antibodies share the same epitope (Figure 4).

As part of the hybridoma screening workflow described above, purified capture antibodies are immobilized in six parallel channels; a screened panel of six high-affinity hybridoma supernatant antibodies is then captured in the channels (Figure 5). Antigen and a second panel of antibodies are then injected into the six orthogonal channels. (The sequence of antibody panels can be exchanged to ensure consistency of results.) The threshold for detection is set from the values for the same antibody (on the diagonal; Figure 6). A negative binding result (dark green in figure) for the second antibody indicates that the hybridoma supernatant antibodies bind competitively to the same antigen epitope.

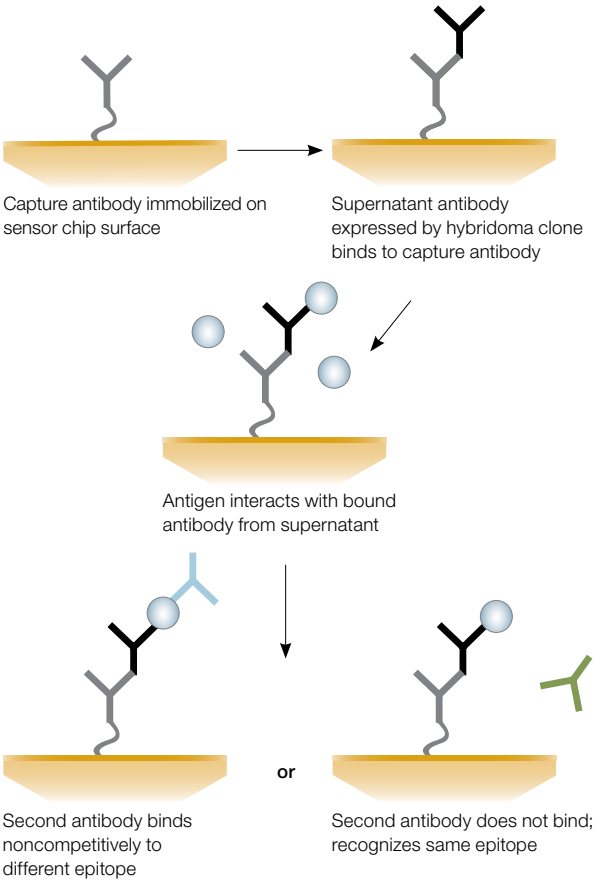


Fig. 4. Representative pairwise epitope mapping workflow. A second panel of antibodies from the supernatant interacts with antigen.

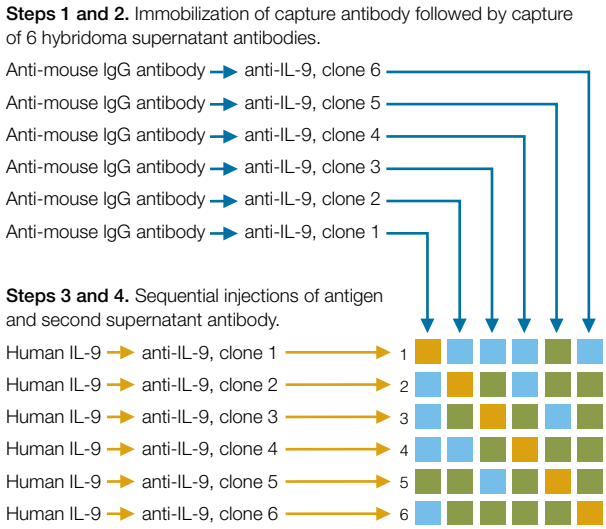


Fig. 5. Pairwise epitope mapping. Representative experimental protocol for epitope mapping. To block all free sites of the immobilized anti-mouse antibody, include an additional step to inject a mouse IgG mixture after interaction of the antigen with the antibody (step 3).

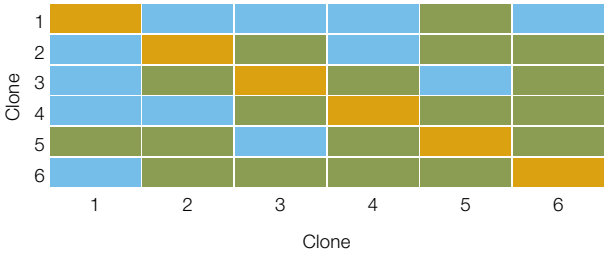


Fig. 6. Representative data matrix showing the interactions among six supernatant antibodies. ■, same epitope, same antibody; ■, same epitope, different antibody; ■, different epitope, different antibody.

Kinetic Analysis of Antibody-Antigen Specificity

Kinetic analysis of antibody-antigen interactions provides essential data for understanding the mechanisms of molecular recognition. During antibody development, the binding specificity of an antibody may be tested by challenge with other analytes (Figure 7). The ProteOn XPR36 interaction array system provides real-time kinetic analysis of 36 label-free antibody-antigen interactions on one sensor chip (Figures 8 and 9) for rapid determination of the kinetic rate constants k_a, k_d, K_A, and K_D.

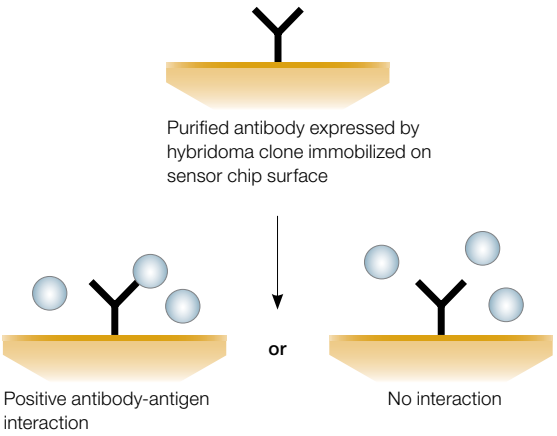


Fig. 7. Representative antibody-antigen specificity analysis workflow. This workflow shows immobilization of purified antibodies from hybridoma supernatants and enables the determination of antibody-antigen specificity and binding kinetics.

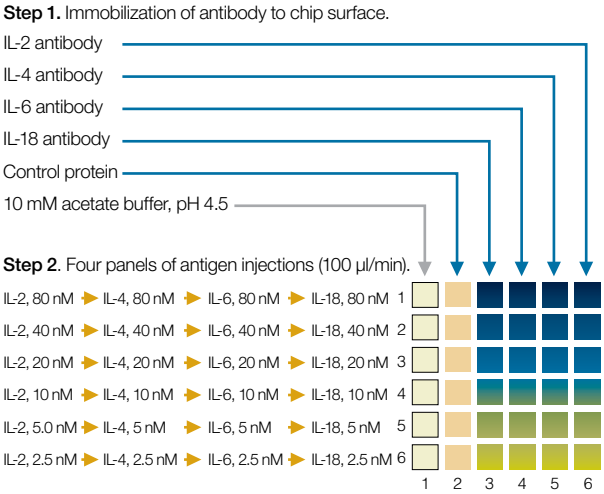


Fig. 8. Multiple antibody-antigen specificity analysis. Representative protocol for analysis of multiple purified antibodies with multiple antigens. Use of hybridoma supernatants requires immobilization of a capture anti-mouse antibody to the chip surface. Each 6 x 6 specificity analysis is followed by sensor chip regeneration.

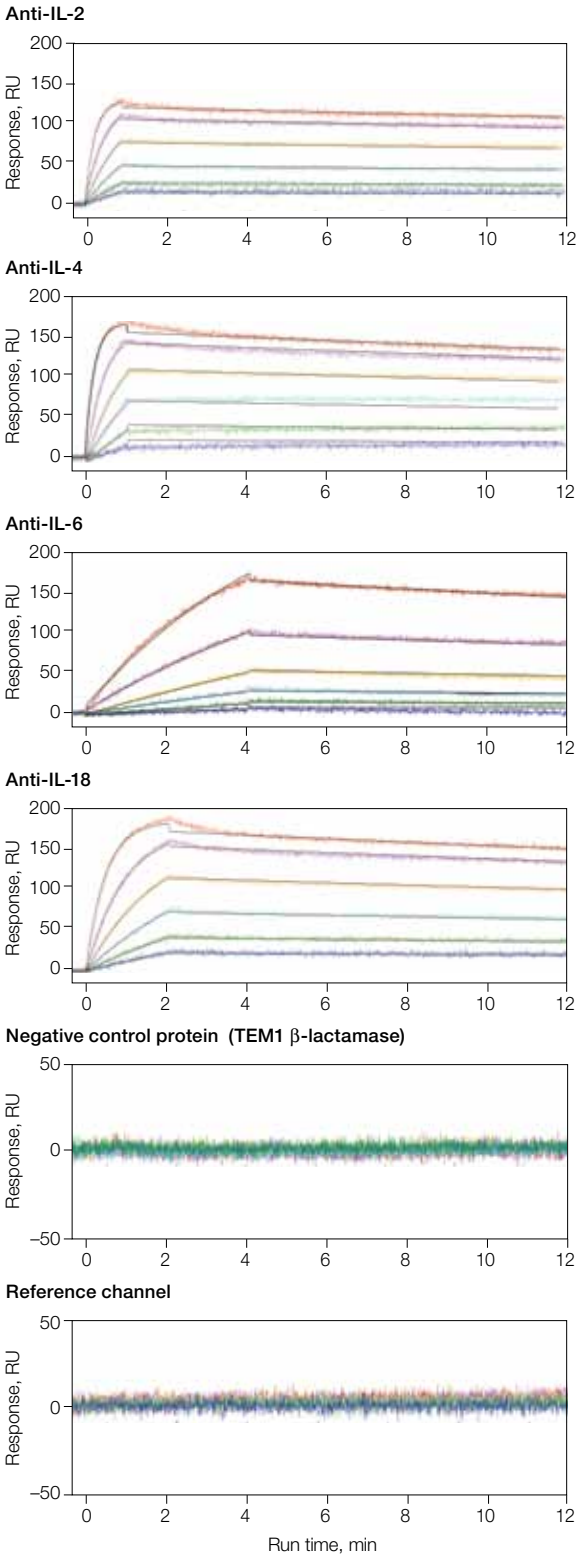


Fig. 9. Four representative sets of six sensorgrams showing global fits to each specific antibody-antigen interaction, with negative control and reference channel plots. For details, see Bronner et al. (2006).

Determination of Cross-Reactivity of Antibodies Used in Multiplex Assays

The complete interaction matrix of a six-member antibody panel can be rapidly determined using the ProteOn XPR36 interaction array system (Figures 10–12). Determining the degree of cross-reactivity among multiple antibodies and antibody clones is important in research, new drug development, and multiplex immunoassays, such as Bio-Plex® magnetic bead assays.

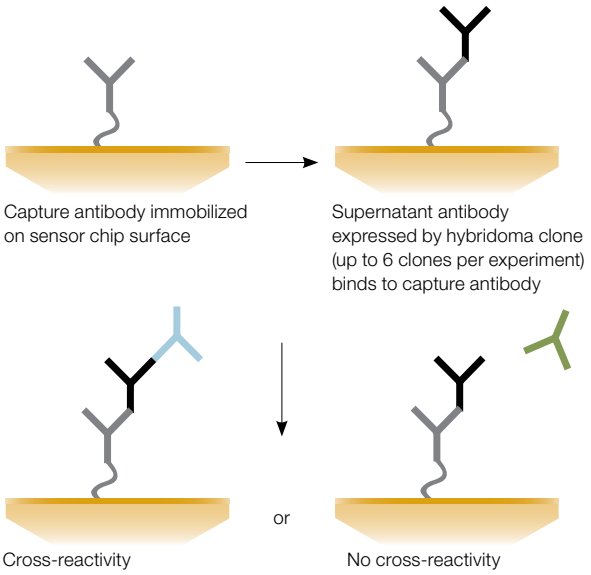


Fig. 10. Representative antibody cross-reactivity analysis workflow. Measurement and analysis of antibody-antibody cross-reactivity.

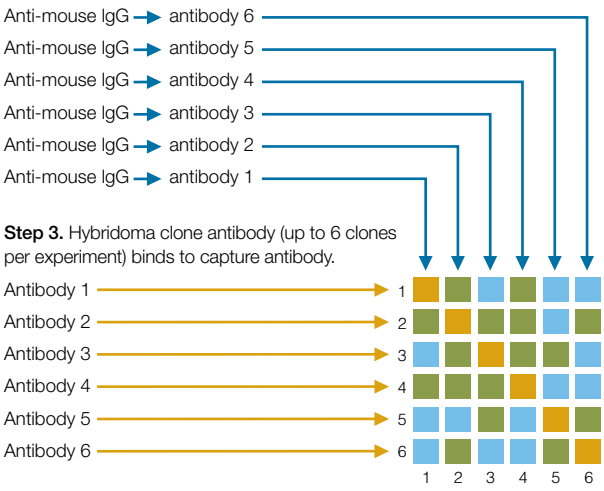


Fig. 11. Determination of cross-reactivity in a panel of six antibodies.

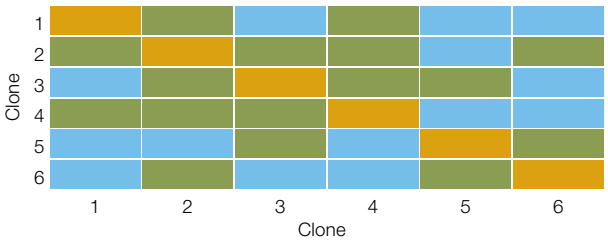


Fig. 12. Representative cross-reactivity matrix showing the interactions among six antibodies. ■, same antibody; ■, no cross-reactivity; ■, cross-reactivity.

Summary

The high-throughput ProteOn XPR36 system is ideally suited for various antibody characterization and development protocols. In the context of large, broad, and very complex studies, for example in which the results of hybridoma screening and ranking data are compared with epitope mapping, specificity analysis, cross-reactivity determination, or other studies, all the advantages of the ProteOn system are multiplied. Multiplex analysis using XPR technology provides the ability to make fast research progress with one instrument. Large amounts of complex data are generated quickly, ready for comparison, and they can be seamlessly integrated and easily categorized.

References

Bronner V et al., Rapid and detailed analysis of multiple antigen-antibody pairs using the ProteOn XPR36 protein interaction array system, Bio-Rad bulletin 5360 (2006)

Bravman T et al., Screening, ranking, and epitope mapping of anti-human IL-9 supernatants, Bio-Rad bulletin 5540 (2007)

Peterlinz KA and Georgiadis R, In situ kinetics of self-assembly by surface plasmon resonance spectroscopy, Langmuir 12, 4731–4740 (1996)

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