Screening, Ranking, and Epitope Mapping of Anti-Human IL-9 Supernatants

Tsafrir Bravman,[1] Vered Bronner,[1] Daniel Laune,[2] Nicolas Novali,[2] Dominique Piquer,[2] Kobi Lavie,[1] and Ariel Notcovich,[1] [1] Bio-Rad Laboratories, Inc., Gutwirth Park, Technion, Haifa 32000, Israel, [2] CNRS UMR 5160, Centre de Pharmacologie et Biotechnologie pour la Santé Faculté de Pharmacie, 34093 Montpellier, Cedex 5, France

Introduction

Monoclonal antibodies have become a particularly important resource for medical research, basic science, and disease diagnosis and therapy. Numerous applications utilizing monoclonal antibodies have been developed, including disease diagnosis, drug delivery to target cells (cancer treatment, immune suppression, HIV treatment), biosensors, organic molecule identification, blood clot detection, and septic shock treatment.

The ProteOn™ XPR36 protein interaction array system is a surface plasmon resonance (SPR) instrument with parallel sample processing capabilities that meets the challenge of high throughput while maintaining all the unique qualities of optical biosensors. The ProteOn system uses a novel microfluidics system to perform simultaneous measurements in an array of 36 interaction spots and 42 local reference spots (interspots). All stages of the experiment, from surface immobilization of "ligand" proteins to their subsequent reaction with "analyte" molecules, are measured in real time to provide comprehensive data for 36 interactions.

Here, we demonstrate the application of the ProteOn XPR36 system to the selection, ranking, and epitope mapping of 20 monoclonal antibody supernatants against human interleukin 9 (IL-9). Anti-mouse antibody was immobilized on a chip and used to capture supernatant antibodies that were further reacted with the IL-9 antigen to determine binding kinetic constants. Four antibodies were then purified, their kinetic rate constants were reanalyzed, and epitope mapping was performed to identify antibodies recognizing different epitopes. We capitalized on the throughput provided by ProteOn XPR36 array technology, capturing five different supernatant antibodies in parallel and then determining their binding kinetic constants in a single injection comprising five antigen concentrations. Repeating this cycle four times allowed the determination of the kinetic parameters of all 20 supernatants within 1 hour.

Methods

Instrumentation and Reagents

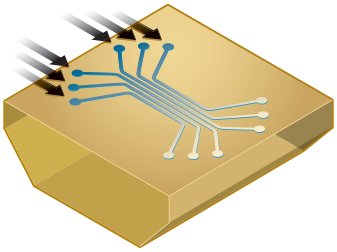
Experiments were performed using the ProteOn XPR36 system. Phosphate buffered saline with 0.005% Tween 20 (PBS/Tween), pH 7.4 was used as running buffer throughout, and all experiments were performed at 25°C. Recombinant human IL-9 was obtained from PeproTech, Inc. Anti-mouse IgG (whole molecule) was obtained from Sigma, and mouse anti-human IL-9 antibody supernatants were obtained by immunizing BALB/c mice with recombinant human IL-9. Hybridomas were obtained after fusion between spleen lymphocytes and Sp2 myeloma cells.

Screening and Ranking of Anti-Human IL-9 Supernatants

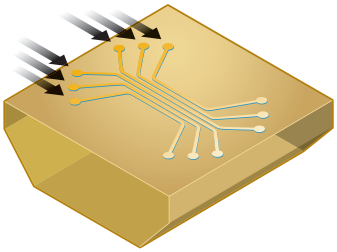
Anti-mouse IgG was immobilized on a ProteOn GLM sensor chip using standard amine coupling chemistry. Using a flow rate of 30 µl/min, the microfluidics network was directed to inject 200 µl of a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) and 0.05 M *N*-hydroxysulfosuccinimide (sulfo-NHS) followed by 200 µl of 90 µg/ml of anti-mouse IgG diluted in 10 mM sodium acetate buffer, pH 4.5 (Figure 1A). The surface was then deactivated with 150 µl of 1 M ethanolamine HCl, pH 8.5 and conditioned using 30 µl of 0.85% phosphoric acid, both at a flow rate of 100 µl/min. About 10,000 RU of anti-mouse IgG was immobilized. Next, five different mouse anti-human IL-9 antibodies (in supernatants) were captured by injecting 150 µl of the supernatants into five of the channels containing immobilized anti-mouse IgG (ligand channels) at a flow rate of 25 µl/min (Figure 1B). A sample of running buffer was injected into the sixth channel, which was used as a reference channel.

At the second step of analyte binding, the microfluidics network directed flow into the six analyte channels orthogonal to the ligand channels (Figure 1C). Five human IL-9 concentrations (6.25, 12.5, 25, 50, and 100 nM) were injected (100 µl) at a flow rate of 50 µl/min. The five analyte concentrations reacted simultaneously with each of the five captured anti-human IL-9 antibodies in a single injection. Running buffer was injected into the sixth channel for double referencing to correct the slow dissociation of the captured antibody from the capturing anti-mouse IgG antibody.

A. Anti-mouse IgG



B. Five mouse anti-human IL-9 supernatants



C. Five concentrations of human IL-9

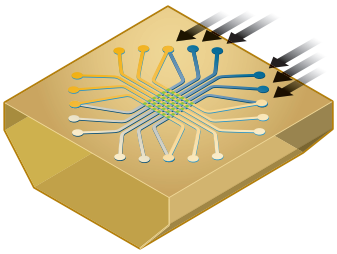


Fig. 1. Reagent flow used for screening and ranking of hybridoma supernatants. A, immobilization of anti-mouse IgG; **B**, capture of five different mouse anti-human IL-9 supernatants (running buffer was injected into the sixth channel); **C**, injection of five concentrations of human IL-9 (running buffer was injected into the sixth channel).

The antibody/antigen complex was removed from the chip by regeneration with 30 µl of 0.85% phosphoric acid at a flow rate of 100 µl/min, allowing the capture of five more anti-human IL-9 antibodies. This workflow was repeated four times until all 20 supernatants were analyzed.

Determining Kinetic Constants of Purified Anti-Human IL-9 Antibodies

Anti-human IL-9 antibodies showing high affinities were purified by affinity chromatography using a protein A support and then immobilized on a ProteOn GLC sensor chip using the amine coupling chemistry described above (EDAC and sulfo-NHS were used for the covalent binding of the purified antibodies to the chip surface). The purified antibodies were diluted with sodium acetate buffer, pH 4.5 to a final concentration of 8 µg/ml and injected into four of the flow channels for 4 min at a flow rate of 30 µl/min. The fifth channel was unmodified and served as a reference channel; the sixth channel remained unused. The chip surface was then deactivated using 1 M ethanolamine HCl, pH 8.5. Ligand densities of 1,000–1,200 RU were achieved. Six concentrations (3.18, 6.25, 12.5, 25, 50, and 100 nM) of human IL-9 were injected (75 µl) into the six channels orthogonal to the channels containing immobilized antibodies at a flow rate of 50 µl/min. The data were analyzed and fitted to a simple 1:1 interaction model.

Epitope Mapping of Anti-Human IL-9 Antibodies

Epitope mapping was performed with the same ProteOn GLC sensor chip used for kinetic analysis directly after regeneration, which was performed with 0.85% phosphoric acid (short pulse of 20 sec). A single concentration of human IL-9 (100 nM) was injected in all channels orthogonal to the immobilized antibodies. The purified antibodies diluted in running buffer were then injected, and the signal at the start of the dissociation was monitored.

Results and Discussion

The objective was to identify high-affinity antibodies that recognize different epitopes on human IL-9. Twenty supernatants containing mouse anti-human IL-9 antibodies were screened and ranked according to their affinities after their kinetic rate constants were determined. Four antibodies showing high affinities were then purified and their kinetic rate constants reanalyzed. Additionally, epitope mapping was performed to identify antibodies recognizing different epitopes.

Screening and Ranking of Anti-Human IL-9 Supernatants

Twenty supernatants containing anti-human IL-9 antibodies developed in mouse were screened to identify positive clones that bound human IL-9 and to determine the kinetic binding constants of the clones. To capture the antibodies from the supernatant, anti-mouse IgG antibody was first immobilized in six channels. In each cycle, five supernatants were captured and their kinetic constants determined. The captured antibodies were then stripped off the chip to allow capture of five additional supernatant antibodies. Overall, four such cycles were performed in about 1 hr, covering the kinetic analysis of all 20 supernatants.

Of the 20 supernatants, four showed no interaction with human IL-9. Some supernatants exhibited complex kinetic behavior that could not be fit to a simple 1:1 interaction model (Figure 2, clone 9). Isotyping studies revealed that these supernatants contained IgM antibodies, which probably accounted for the deviation from the simple Langmuir interaction (not shown). The kinetic rate constants of all other

supernatants were determined, and representative results from five of the antibodies are summarized in Table 1; their fits are shown in Figure 2. The five selected supernatants had similar affinities, though supernatant 2 had a slightly higher affinity (lower equilibrium dissociation constant, K_D) and a relatively lower dissociation rate constant than the others.

Table 1. Kinetic analysis and ranking* of anti-human IL-9 supernatants of selected clones.

Supernatant	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}

* Highest to lowest affinity, in descending order.

Kinetic Analysis of Purified Anti-Human IL-9 Antibodies

After screening and ranking of the 20 supernatants, four anti-human IL-9 antibodies were chosen for further purification: clones 1 and 2 and two subclones of parent clone 7 (termed 7a and 7b). These purified antibodies were then subjected to detailed kinetic analysis. The four antibodies were immobilized on a ProteOn GLC sensor chip using standard amine coupling. Samples of human IL-9 analyte were prepared at six different concentrations and injected simultaneously over the four antibodies; an empty reference channel was also included. Consequently, a single injection generated six concentration-dependent sensorgrams for each antibody, allowing full kinetic rate constant determination (Figure 3). The data were fitted to a simple 1:1 interaction model, and the kinetic constants thus derived are summarized in Table 2. All four antibodies had equilibrium dissociation constants in the nanomolar range; however, antibodies from clones 1 and 2 had slower dissociation rate constants than subclones 7a and 7b, indicating that the antibody-antigen complexes of antibodies 1 and 2 were more stable.

The kinetic constants derived from the analysis of purified antibodies were comparable to those obtained from supernatants, demonstrating that valuable information can be obtained using unpurified antibodies in supernatants.

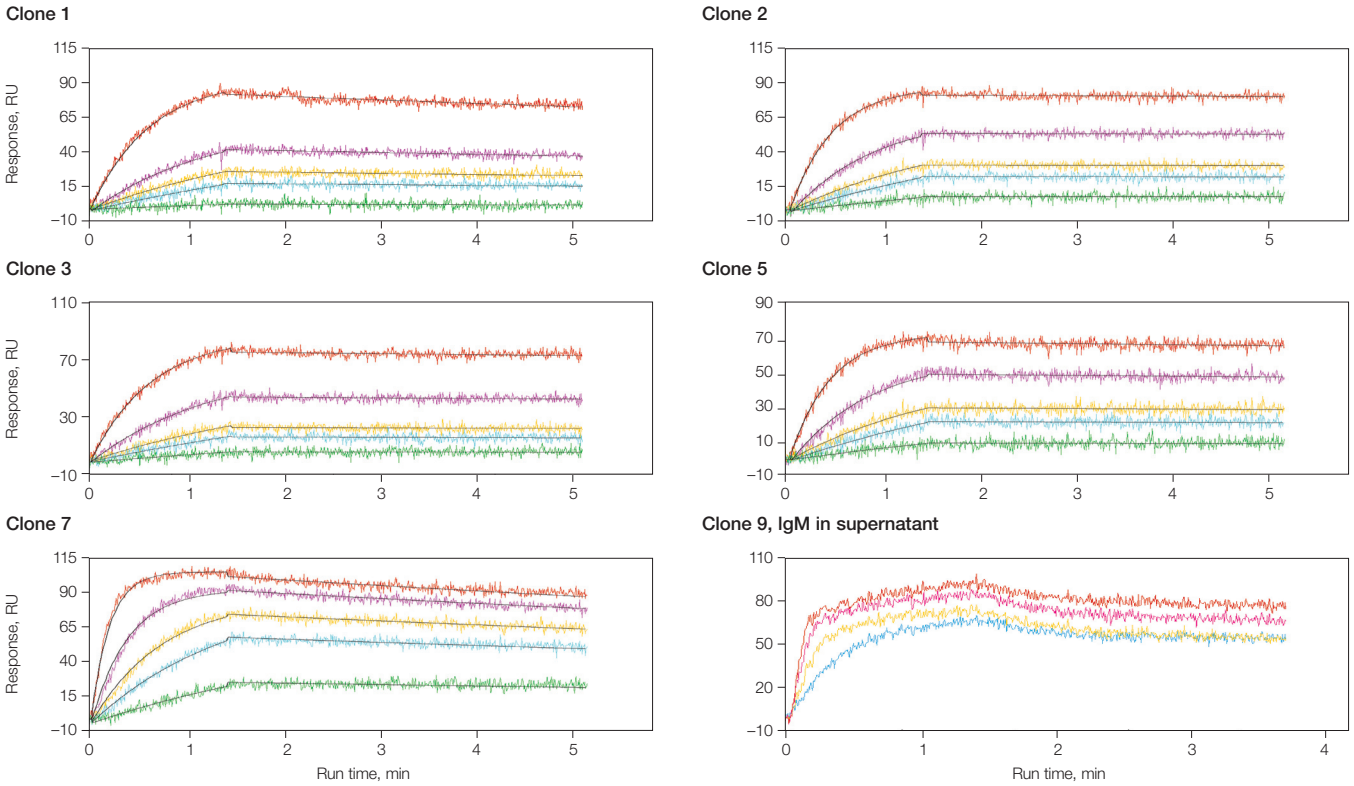


Fig. 2. Screening of mouse anti-human IL-9 supernatants. Shown are sensorgrams generated by the interactions between six different anti-human IL-9 supernatants with five concentrations (—, 100 nM; —, 50 nM; —, 25 nM; —, 12.5 nM; —, 6.25 nM) of human IL-9. Black lines represent the global fit of the sensorgrams to a 1:1 interaction model. See Table 1 for the kinetic rate constants derived from these data.

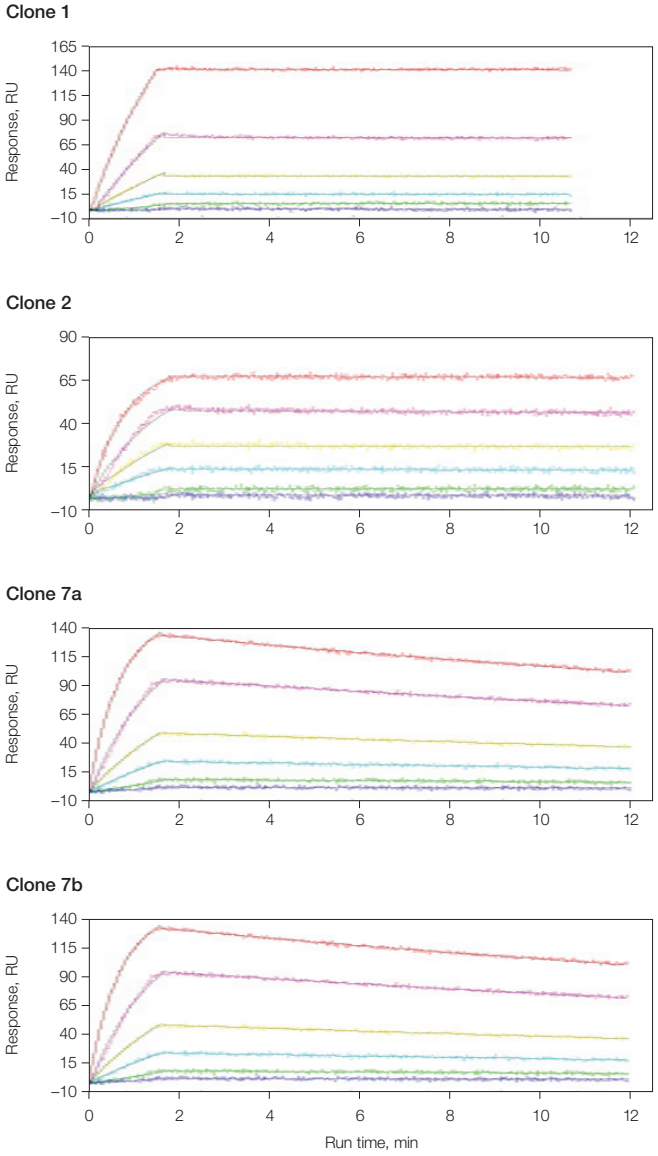


Fig. 3. Kinetic analysis of the IL-9 antibody/IL-9 interaction. Shown are sensorgrams generated by the interaction of four purified anti-human IL-9 antibodies with six concentrations of human IL-9 (—, 100 nM; —, 50 nM; —, 25 nM; —, 12.5 nM; —, 6.25 nM; —, 3.18 nM). Black lines represent the global fit of the sensorgrams to a 1:1 interaction model. See Table 2 for the kinetic rate constants derived from these data.

Epitope Mapping of Purified Anti-Human IL-9 Antibodies

In the next step, epitope mapping of the four purified antibodies was performed to determine if these antibodies recognized different or similar epitopes. For this purpose, the same ProteOn GLC chip containing the four immobilized antibodies was used. A single concentration of human IL-9 was injected in the analyte orientation (over the four immobilized antibodies), resulting in formation of antibody-antigen complexes. This step was followed by an immediate injection of the four antibodies (also in the analyte orientation), forming a sandwich complex of antibody-antigen-antibody. The level of binding of the second set of antibodies was monitored at the start of the dissociation phase, and the values obtained are summarized in Table 3. All values were normalized according to the signals obtained when the same antibody was used both as the first and second antibody.

The results indicate that antibodies 1 and 2 shared the same or overlapping epitopes, since no binding was observed when these two were tested pairwise. The same was true for antibodies 7a and 7b; however, the epitope recognized by antibodies 1 and 2 was different from that recognized by 7a and 7b (Table 3). Therefore, these two epitopes were located at distinct places on the human IL-9 antigen (Figure 4).

Table 2. Kinetic analysis of purified anti-human IL-9 antibodies.

Clone	k_a ($M^{-1}sec^{-1}$)	k_d (sec^{-1})	K_D (M)
1	5.3×10^4	9.2×10^{-5}	1.7×10^{-9}
2	7.1×10^4	6.3×10^{-5}	8.9×10^{-10}
7a	2.0×10^5	7.9×10^{-4}	4.0×10^{-9}
7b	1.9×10^5	5.6×10^{-4}	2.9×10^{-9}

Table 3. Epitope mapping of purified anti-human IL-9 antibodies. Values represent signals observed at the start of the dissociation phase after normalizing against signals observed when the same antibody was used as both the first and the second antibody. Colors represent antibody pairs (interacting once as the first antibody and once as the second antibody).

		First Antibody			
		2	1	7a	7b
Second Antibody	2	1	**0.2**	5	6
	1	**0.3**	1	3	4
	7a	49	27	1	**1**
	7b	44	19	**1**	1

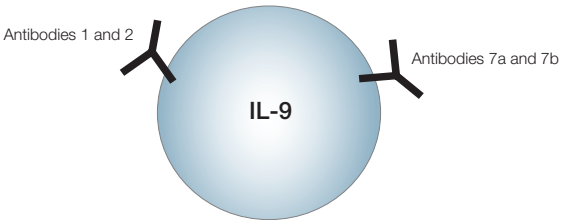


Fig. 4. Representation of the results of epitope mapping. Antibodies 1 and 2 share the same or overlapping epitopes, as do antibodies 7a and 7b. However, the epitope recognized by antibodies 1 and 2 is different from that recognized by 7a and 7b.

Conclusions

This study demonstrates the rapid, precise screening and ranking of 20 hybridoma supernatants using the ProteOn XPR36 system. Five high-affinity antibodies were identified, and four of these were selected for further purification and epitope mapping. Two of the antibodies recognized different epitopes on the IL-9 antigen. Both epitope mapping and hybridoma ranking were accomplished efficiently using the parallel processing capability of the ProteOn XPR36 system. This work can be easily enhanced to include kinetic constant determination of hundreds of supernatants within only a few hours.

In the workflow used in this study, five supernatants were screened at a time against five antigen concentrations, and one channel was reserved for referencing. Using this configuration, both the anti-mouse IgG and antibody-containing supernatants were injected into the same set of channels (ligand channels), followed by IL-9 injection into the orthogonal analyte channels (Figure 1). New applications being tested, however, have shown that by using a different configuration for sample flow and interspot referencing, all six available analyte channels may be used for supernatant screening, yielding equally reliable results with greater throughput. This is accomplished by using the ligand channel for immobilization of anti-mouse IgG, the analyte channel for capture of the supernatant clones, and the ligand channels for IL-9 flow. For more information, refer to the ProteOn XPR36 Application Guide, bulletin 5412.

Information in this tech note was current as of the date of writing (2006) and not necessarily the date this version (rev A, 2007) was published.

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