Rapid Screening and Selection of Optimal Antibody Capturing Agents Using the ProteOn™ XPR36 Protein Interaction Array System

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Introduction

The production of antibodies for use as research tools or for diagnostic and therapeutic applications is a major focus for many life science researchers. Immunoglobulin (Ig) binding proteins of bacterial origin have long been used for purification of antibodies, and for the detection of those antibodies in immunological assays. Each of these Ig binding proteins has different antibody-binding properties in terms of the portion of the antibody that is recognized, as well as the species and class of antibodies it will bind. Proteins A, G, A/G, and L are native and recombinant proteins that bind almost exclusively with the IgG class of antibodies and are widely available commercially. Protein A is generally preferred for rabbit, pig, dog and cat IgG. Protein G has better binding capacity for a broader range of mouse and human IgG subclasses (IgG1, IgG2, etc.). Protein A/G is a recombinant fusion protein that includes the IgG-binding domains of both protein A and protein G and binds a very broad range of IgG subclasses from rabbit, mouse, human, and other mammalian samples. Protein L binds to certain immunoglobulin κ light chains, which occur in all classes of Igs (i.e., IgG, IgM, IgA, IgE, and IgD). Only those antibodies within each class that possess the appropriate κ light chains will bind. For any given antibody screening and/or purification, empirical testing is best used to determine which of these antibody-binding proteins is best suited to the application, and knowing the strength of binding of each of these proteins to the antibody(ies) of interest will aid in that determination.

The ProteOn XPR36 protein interaction array system and the One-shot Kinetics™ technology can be used for the rapid screening and selection of antibody-binding proteins that provide the optimal binding characteristics for a particular application. This system allows multiple quantitative protein binding analyses in parallel, providing association, dissociation, and affinity constants for several antibody-binding proteins simultaneously. The system integrates a unique 6 x 6 interaction array for the analysis of up to six ligands and up to six analytes, producing 36 data points in a single experiment. Because multiple conditions can be tested in parallel, comprehensive kinetic analysis of an antibody concentration series can be handled in one experiment, without the need for regeneration steps.

In this tech note, we demonstrate an efficient, rapid method for determining the optimal antibody-binding protein to use for screening and/or purification of antibodies of interest using the ProteOn XPR36 protein interaction array system. The entire experiment is performed using only one ProteOn sensor chip.

Methods

Instrumentation and Reagents

All experiments were performed using the ProteOn XPR36 protein interaction array system and ProteOn GLM sensor chips, all from Bio-Rad Laboratories, Inc. Phosphate buffered saline, pH 7.4 with 0.005% Tween 20 (PBST) was used as running buffer throughout and all experiments were performed at 25°C.

Immobilization of Ig-Binding Proteins

Immobilization of the four Ig-binding proteins was performed simultaneously using the parallel sample processing capability of the ProteOn XPR36 system, and was accomplished in only three injection steps for surface activation, ligand coupling, and surface deactivation. The ProteOn GLM sensor chip surface was activated for 1 min (flow rate 30 µl/min) in four of the six ligand channels by injecting a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, 10 mM) and *N*-hydroxysulfosuccinimide (sulfo-NHS, 2.5 mM), which are components of the ProteOn amine coupling kit. The last two channels were not modified in this step, allowing them to serve as reference channels.

The four Ig-binding proteins were diluted to the following concentrations:

- Protein A (Sigma-Aldrich, Inc.), 25 µg/ml in 10 mM ProteOn acetate buffer, pH 4.5
- Protein A/G (Thermo Fisher Scientific Inc.), 25 µg/ml in 10 mM ProteOn acetate buffer, pH 4.0
- Protein L (Sigma-Aldrich, Inc.), 100 µg/ml in 10 mM ProteOn acetate buffer, pH 3.5
- Protein G (Sigma-Aldrich, Inc.), 25 µg/ml in 10 mM ProteOn acetate buffer, pH 3.0

Immobilization of the four Ig-binding protein samples (150 µl) was performed by injection into the four activated ligand channels at a flow rate of 30 µl/min. Deactivation of the unreacted carboxyl groups on the GLM sensor chip was achieved by injection of 150 µl of 1 M ethanolamine HCl, pH 8.5.

Interaction of Antibody Subclasses With Ig-Binding Proteins

Antibody samples were all prepared by serial dilution in PBS/Tween. Mouse IgG2$_a$κ, mouse IgG2$_b$κ, mouse IgG2λ, mouse IgG1κ, human IgG2κ, human IgG3κ (Sigma-Aldrich, Inc.), and human IgG1λ (Applied Immune Technologies) were prepared at concentrations of 10, 5, 2.5, 1.25, 0.625, and 0.3125 nM. Each concentration series of an antibody was injected into the six analyte channels orthogonal to the ligand channels (containing Ig-binding proteins) at a flow rate of 50 µl/min, for 6 min. These ligand channels were regenerated between injections of each antibody concentration series by a short pulse (30 µl) of 10 mM glycine, pH 2.0.

Capture of a Model Antibody

A human IgG1λ antibody was used as a model to compare binding of its antigen to IgG1λ captured either by the Ig-binding proteins or immobilized directly to the sensor chip. Capture of the human IgG1λ by the Ig-binding proteins was performed by injecting 100 µl of the antibody (10 µg/ml in PBST) at a flow rate of 30 µl/min into the four ligand channels containing the Ig-binding proteins. Human IgG1λ was also bound directly to the fifth ligand channel (no Ig-binding protein used) of the GLM sensor chip by standard amine coupling, using the following conditions: activation with a mixture of 3.33 mM EDAC and 0.83 mM sulfo-NHS for 1 min (30 µl/min), injection of human IgG1λ (10 µg/ml in 10 mM Na acetate pH 4.5) for 2.5 min (30 µl/min), and blocking with 1 M ethanolamine pH 8.5 for 5 min. The sixth channel was not modified and served as a reference channel.

Kinetic Binding Analysis of the Model Antibody With its Target Antigen

Samples of a target antigen (a biomarker for skin cancer) provided by Applied Immune Technologies were prepared at concentrations of 1,000, 50, 250, 125, and 62.5 nM by serial dilution in PBST. Samples of each concentration (50 µl) were injected into the five analyte flow channels at a flow rate of 50 µl/min. Running buffer (PBST) was injected into the sixth channel for double referencing to correct for the slow dissociation of the captured antibody from the Ig-binding proteins.

Data Analysis

The data were analyzed with the ProteOn Manager™ 2.0 software. Binding curves were processed for baseline and start injection alignment, together with interspot reference subtraction. Each set of six reference-subtracted sensorgrams was fitted globally to curves describing a homogeneous 1:1 bimolecular reaction model. Each ligand surface interacting with its different analyte concentrations was grouped together to fit the k_a, k_d, and R_{max} parameters, and the equilibrium dissociation constant, KD, was calculated using the equation $KD = k_d/k_a$.

Results and Discussion

Immobilization of Ig-Binding Proteins

The four Ig-binding proteins were immobilized using standard amine coupling on a ProteOn GLM sensor chip. Each protein requires different immobilization conditions, including pH and concentration, in order to attain optimal ligand densities and activity levels and thus provide reliable kinetic binding curves and analysis. These conditions and the immobilization levels are summarized in Table 1. Since the flow rate and injection times were identical for all of the Ig-binding proteins, the four immobilizations could be performed in parallel. The total time required to immobilize the four Ig-binding proteins was approximately 20 minutes (including washing of the needles between the different stages).

Binding Analysis of the Interaction of Ig-Binding Proteins With Different Antibody Subclasses

In general, antibodies are divided into five different classes: IgG, IgM, IgA, IgE, and IgD. Minor differences within the IgG class further divide this group into four subclasses: IgG1, IgG2$_a$, IgG2$_b$ and IgG3. In addition, each subclass can have either κ or λ light chain. The interactions between the four subclasses of IgG antibodies from both mouse and human and the four Ig-binding proteins were analyzed. Specifically,

Table 1. Immobilization conditions and ligand densities.

	EDAC/Sulfo-NHS	Ig-Binding Protein		Ethanolamine-HCl		Ligand Density,
	Concentration, mM/mM	Concentration, µg/ml	pH	Concentration, M	pH	RU
Protein A	6.66/1.66	25	4.5	1	8.5	2,000
Protein A/G	6.66/1.66	25	4.0	1	8.5	2,200
Protein L	20/5	100	3.5	1	8.5	2,500
Protein G	13.33/3.33	25	3.0	1	8.5	500

the binding kinetics of seven different antibodies were determined, including mouse IgG2$_a$κ, mouse IgG2$_b$κ, mouse IgG2$_a$κ, mouse IgG1κ, human IgG2κ, human IgG1κ, and human IgG3κ.

The binding interactions were fit to a 1:1 Langmuir binding model, as shown in Figure 1. The kinetic binding constants are summarized in Table 2. Affinities (kD) ranged from several nM to very low sub nM values, primarily because dissociation rates were very slow (<1x10^{-6} sec^{-1}).

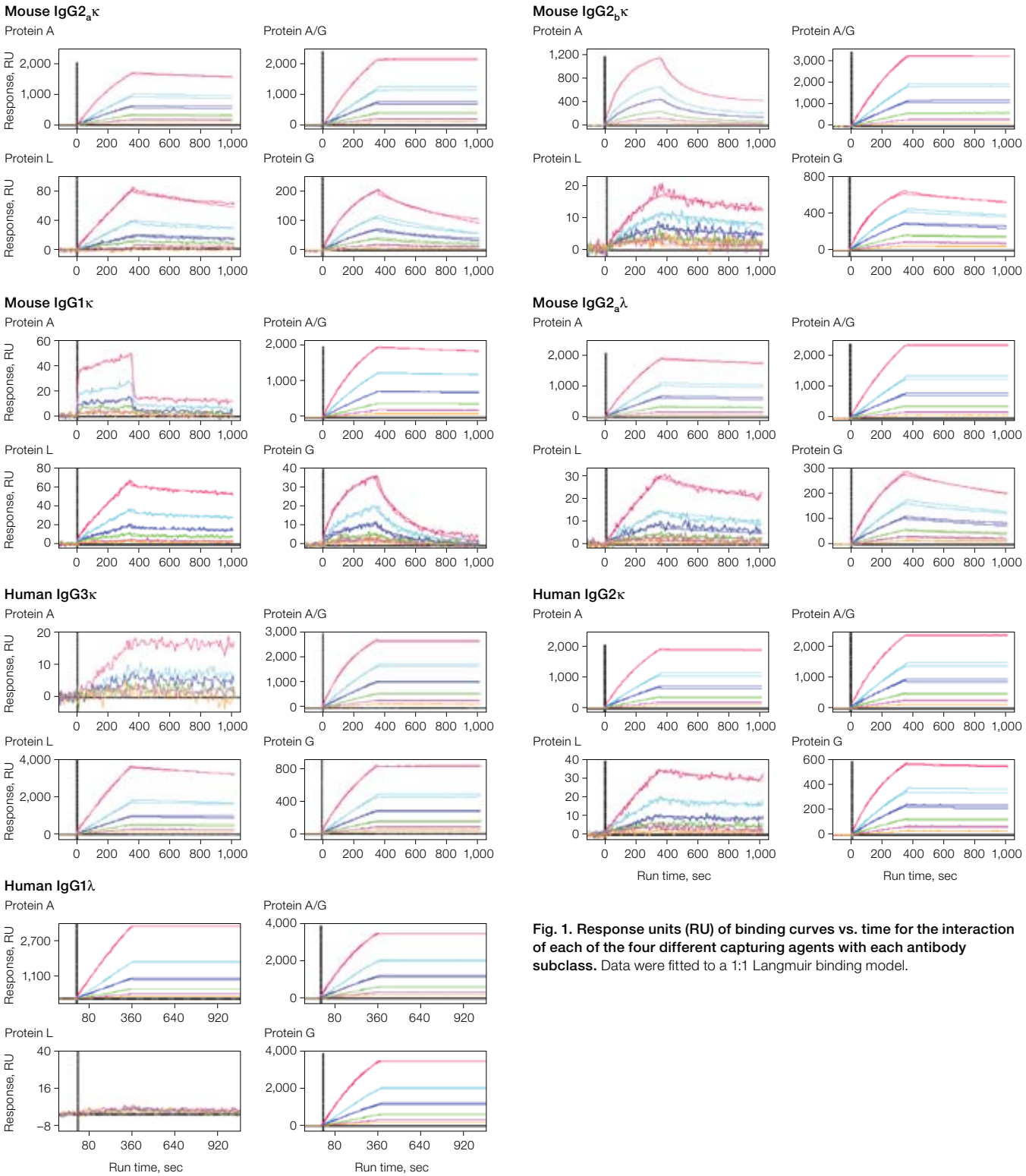

Fig. 1. Response units (RU) of binding curves vs. time for the interaction of each of the four different capturing agents with each antibody subclass. Data were fitted to a 1:1 Langmuir binding model.

Table 2. Kinetic binding constants of the Ig-binding proteins interacting with different antibody subclasses.

	Antibody	k_a, $M^{-1}s^{-1}$	k_d, s^{-1}	KD, M
Mouse $IgG2_a\kappa$	Protein A	2.62×10^5	1.02×10^{-4}	4.01×10^{-10}
	Protein A/G	2.09×10^5	$<1.0 \times 10^{-6}$	$<1.0 \times 10^{-11}$
	Protein L	8.87×10^3	5.00×10^{-4}	5.64×10^{-8}
	Protein G	3.56×10^5	1.02×10^{-3}	2.92×10^{-9}
Mouse $IgG2_b\kappa$	Protein A	No 1:1	No 1:1	No 1:1
	Protein A/G	3.69×10^5	$<1.0 \times 10^{-6}$	$<1.0 \times 10^{-11}$
	Protein L	4.28×10^5	5.14×10^{-4}	1.22×10^{-9}
	Protein G	6.62×10^5	2.57×10^{-4}	3.96×10^{-10}
Mouse $IgG2_a\lambda$	Protein A	2.33×10^5	1.16×10^{-4}	5.08×10^{-10}
	Protein A/G	2.01×10^5	$<1.0 \times 10^{-6}$	$<1.0 \times 10^{-11}$
	Protein L	2.15×10^4	6.22×10^{-4}	2.91×10^{-8}
	Protein G	3.54×10^5	4.39×10^{-4}	1.24×10^{-9}
Human IgG2κ	Protein A	2.87×10^5	1.01×10^{-5}	3.55×10^{-11}
	Protein A/G	3.11×10^5	$<1.0 \times 10^{-6}$	$<1.0 \times 10^{-11}$
	Protein L	2.02×10^4	2.83×10^{-4}	1.36×10^{-8}
	Protein G	4.01×10^5	2.78×10^{-5}	6.70×10^{-11}
Mouse IgG1κ	Protein A	—	—	—
	Protein A/G	3.29×10^5	6.83×10^{-5}	2.08×10^{-10}
	Protein L	9.95×10^4	2.66×10^{-4}	2.68×10^{-9}
	Protein G	1.93×10^5	4.39×10^{-3}	2.64×10^{-8}
Human IgG3κ	Protein A	—	—	—
	Protein A/G	5.13×10^5	$<1.0 \times 10^{-6}$	$<1.0 \times 10^{-11}$
	Protein L	2.74×10^5	2.64×10^{-4}	1.18×10^{-9}
	Protein G	3.16×10^5	$<1.0 \times 10^{-6}$	$<1.0 \times 10^{-11}$
Human IgG1λ	Protein A	5.49×10^4	$<1.0 \times 10^{-6}$	$<1.0 \times 10^{-10}$
	Protein A/G	2.09×10^5	$<1.0 \times 10^{-6}$	$<1.0 \times 10^{-11}$
	Protein L	—	—	—
	Protein G	2.04×10^4	$<1.0 \times 10^{-6}$	$<1.0 \times 10^{-11}$

Three interactions however, could not be fitted to the theoretical curve since the signal levels were very low for the binding of protein A with mouse IgG1κ and human IgG3κ, and for protein L with human IgG1λ. Additionally, the interaction between protein A and mouse $IgG2_b\kappa$ could not be fitted with the 1:1 model, presumably because the interaction is more complex.

The affinity constants derived from the ProteOn system were compared to relative binding strength values reported by Pierce protein research products (Thermo Scientific), as shown in Table 3. The most notable observation from this table is the weak interaction of Protein A with mouse IgG1κ and human IgG3κ, which is in agreement with the Pierce protein products data. These interactions produced very low signals with the ProteOn system, as would be expected for very low affinity interactions. Additionally, the weaker affinity, as measured with the ProteOn system, of the IgG1κ for protein G, compared to the affinity of the other Ig subclasses for protein G, is in good accordance with the reported results. The same is true for mouse IgG1κ interacting with protein A/G. Finally, we did not observe any binding of human IgG1λ with protein L, again in accordance with the data from Pierce protein products.

Antibody Capture and Interaction With Antigen

A cancer biomarker target antigen and its antibody were selected as a model to demonstrate its efficiency of capture by each of the four Ig-binding proteins and subsequently its interaction with its target antigen. The subclass of the antibody was human IgG1λ. The levels captured by the four Ig-binding proteins were: protein A 3,500 RU; protein A/G 3,000 RU; protein G 1,000 RU; protein L, no binding; and 1,200 RU for direct, covalent immobilization of the antibody to the sensor chip by amine coupling.

After capture of the antibody, five concentrations of the target antigen were injected in parallel using the One-shot Kinetics approach. Running buffer was injected into the sixth channel in order to correct for the decay of the captured antibody from the Ig-binding proteins. In this manner, the kinetic binding constants for the interaction between the antigen and the antibody captured by each of the four Ig-binding proteins, as well as the affinity of the antigen for the immobilized sensor chip surface in the absence of an Ig-binding protein (control), could be determined in a single injection. The binding curves are presented in Figure 2, and the binding constants are summarized in Table 4. All the k_a and k_d values were very similar, indicating that the capture agent had no effect on the binding kinetics for the interaction of the antibody with its antigen.

Most important however is how well each Ig-binding protein maintained the ability of the captured antibody to bind its antigen. The activity of the captured antibody is determined by first calculating the theoretical maximum response (R_{max}), if every captured antibody molecule bound its antigen. This is determined using the following formula:

$$\text{Theoretical } R_{max} = \frac{MW_A}{MW_L} \times L \times n$$

Where MW_A is molecular weight of the analyte and MW_L is molecular weight of the ligand. L is the ligand density in RU and n is the stoichiometry of the interaction. In this case the MW of analyte is 50 kD, and the MW of captured antibody is 150 kD. Each captured antibody can then bind 2 molecules of analyte (stoichiometry of 2). When the IgG binding RU value (ligand density) obtained for each Ig-binding protein is inserted into this equation, the result is the theoretical R_{max} shown in Table 4.

Table 3. Affinity constants of the interactions between the different antibodies and the Ig-binding proteins.

Antibody	SPR, M	Protein G*	SPR, M	Protein L*	SPR, M	Protein A/G*	SPR, M	Protein A*
Mouse $IgG2_a\kappa$	2.92×10^{-9}	+++	5.64×10^{-8}	+++	1.0×10^{-11}	+++	4.01×10^{-10}	+++
Mouse $IgG2_a\lambda$	1.24×10^{-9}	+++	2.91×10^{-8}	+++	1.0×10^{-11}	+++	5.08×10^{-10}	+++
Mouse $IgG2_b\kappa$	3.96×10^{-10}	+++	1.22×10^{-9}	+++	1.0×10^{-11}	+++	No fit	+++
Human IgG2κ	6.70×10^{-11}	+++	1.36×10^{-8}	+++	1.0×10^{-11}	+++	3.35×10^{-11}	+++
Mouse IgG1κ	2.64×10^{-8}	++	2.68×10^{-9}	+++	2.08×10^{-10}	++	—	+
Human IgG3κ	$<1.0 \times 10^{-11}$	+++	1.18×10^{-9}	+++	1.0×10^{-11}	+++	—	+
Human IgG1λ	$<1.0 \times 10^{-11}$	+++	—	—	1.0×10^{-11}	+++	$<1.0 \times 10^{-10}$	+++

* Data for relative affinities were taken from the Thermo Fischer Scientific website (http://www.piercenet.com/files/TR0034dh4-Ab-binding-proteins.pdf; www.thermo.com/pierce).

Table 4. Capture levels, binding kinetics with the antigen, and activity of the captured antibody.

Capturing Agent	IgG Binding, RU	k_a, $M^{-1}s^{-1}$	k_d, s^{-1}	KD, M	R_{max}, RU	Theoretical R_{max}	% Activity
Protein A	3,500	1.36×10^5	1.23×10^{-1}	9.02×10^{-7}	424	2,333	18
Protein A/G	3,000	1.45×10^5	1.20×10^{-1}	8.31×10^{-7}	518	1,922	26
Protein G	1,000	1.32×10^5	1.23×10^{-1}	9.35×10^{-7}	423	660	64
Amine Coupling	1,200	1.57×10^5	1.47×10^{-1}	9.37×10^{-7}	57	814	7

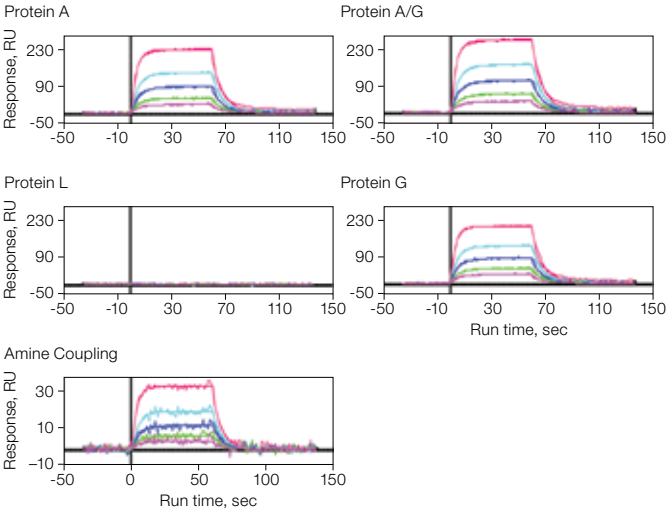

Fig. 2. Interaction of the target antigen with its antibody. The curves for the binding of the target antigen to antibody captured by each of the Ig-binding proteins, or by antibody bound directly to the sensor chip (amine coupling), are shown.

Once the R_{max} is known, the activity of each captured antibody for its antigen can be determined by the following formula:

$$\% \text{ activity} = (\text{Observed } R_{max}/\text{Theoretical } R_{max}) \times 100$$

Table 4 shows that antibody captured by protein G has the highest activity for the antigen analyte (64%), although it has one of the lower binding densities to the sensor chip (1,000 RU). Even so, the observed antigen binding signals (R_{max}) were similar to those when more than 3,000 RU were captured by protein A, which was only 18% active. Covalent immobilization of the antibody to the sensor chip surface resulted in very low antigen-binding activity (7%), suggesting that most of the antibody molecules immobilized in this fashion were incapable of binding the antigen.

In general, the best antibody capture agent is the one that maintains the highest activity of the captured antibody, does not change the binding kinetics between the antibody and its antigen, and makes the most stable interaction with the captured antibody (slowest k_d rate), provided that a significant amount of antibody is captured. The kinetic constant values for the binding of the model antibody and its antigen are very similar regardless of the Ig-binding protein used (Table 4). The k_ds for the interaction of each Ig-binding protein with human IgG1λ are also very similar (Table 2). Therefore protein G is the optimal capture agent to use with this model antibody, due to its ability to maintain high activity of the antibody.

Conclusion

The ProteOn XPR36 protein interaction array system and the One-shot Kinetics approach can be valuable tools for the selection of the appropriate Ig-binding protein to use when analyzing and/or purifying antibodies. In addition, they can provide insight into the nature of the binding interaction between the antibody and its antigen (Nahshol et al. 2008). Several Ig-binding proteins can be rapidly assessed simultaneously in one experiment and the best candidate be chosen based on both its ability to capture the antibody of interest, and its proficiency for preserving the ability of the antibody to bind to its antigen.

Reference

Nahshol et al. (2008) Rapid kinetic analysis and affinity determination of hundreds of monoclonal antibodies. Anal. Biochem 383, 52-60.

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