Rapid Optimization of Immobilization and Binding Conditions for Kinetic Analysis of Protein-Protein Interactions Using the ProteOn™ XPR36 Protein Interaction Array System

Vered Bronner,[1] Tsafrir Bravman,[1] Ariel Notcovich,[1] Dana Reichmann,[2] Gideon Schreiber,[2] and Kobi Lavie[1]

[1] Bio-Rad Laboratories, Inc., Gutwirth Park, Technion, Haifa 32000, Israel
[2] Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

Introduction

The characterization of protein-protein interactions is a fundamental need in the rapidly growing field of proteomics. Surface plasmon resonance (SPR) technology is widely used for kinetic studies of protein-protein interactions. In the design of SPR experiments, however, determining the optimal conditions for ligand immobilization and analyte binding can often require some trial and error.

The ProteOn XPR36 protein interaction array system is an SPR biosensor with a multichannel module and interaction array sensor chip for analysis of up to 36 protein interactions in a single injection step. The parallel sample processing capability of the ProteOn XPR36 system can facilitate the determination of optimal reaction conditions. Because multiple conditions can be tested in parallel and in a single injection step, the effects of each condition on an interaction can be viewed in context of all the conditions simultaneously. Thus, the information needed to make progress toward a successful result is generated more quickly and with more flexibility than is possible with serial sample analysis. Additional details on this array-format system are provided in Bronner et al. (2006).

In this tech note, we demonstrate an efficient, rapid protocol for determining the optimal ligand immobilization and analyte binding conditions for a model protein interaction using the ProteOn XPR36 system. We then use these conditions to perform extensive kinetic and equilibrium analyses of protein-protein binding. The entire experiment is performed in just two ligand immobilization and analyte binding cycles using just two ProteOn sensor chips. In the first cycle, multiple conditions are tested, and in the second cycle, a detailed kinetic analysis is performed under the conditions found to be optimal in the first cycle.

Methods

Instrumentation, Reagents, and Experimental Model

Experiments were performed using the ProteOn XPR36 protein interaction array system and two ProteOn GLC sensor chips. ProteOn phosphate buffered saline with 0.005% Tween 20, pH 7.4 (PBS/Tween) was used as running buffer, and all experiments were performed at 25°C.

The experimental model used consisted of wild-type TEM1β-lactamase (TEM1, pI = 5.0) and a mutant form of β-lactamase inhibitor protein (BLIP-F142A) (Albeck and Schreiber 1999, Bush 1989).

Optimization of Ligand Immobilization and Analyte Binding Conditions

In the first injection cycle, the optimal pH for TEM1 immobilization was investigated. TEM1 protein was immobilized onto five of the six ligand channel surfaces using the amine coupling reagents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC, 133 mM) and *N*-hydroxysulfosuccinimide (sulfo-NHS, 33 mM) (components of the ProteOn amine coupling kit). The sixth channel was not modified and served as a reference channel. TEM1 samples (2 µM) were prepared in 10 mM ProteOn acetate buffer with pH values of 3.0, 3.5, 4.0, 4.5, and 5.0. The five TEM1 samples (180 µl) were injected in parallel at a flow rate of 30 µl/min. To deactivate remaining surface groups in the five activated channels, 1 M ethanolamine HCl, pH 8.5 (ProteOn amine coupling kit) was then injected. A sample of running buffer was included in each step for injection into the reference channel.

For analyte (BLIP-F142A) binding, the multichannel module directed flow into the six parallel analyte channels orthogonal to the six ligand (TEM1) channels. Six solutions of BLIP-F142A were prepared at concentrations of 600, 300, 150, 75, 37.5, and 18.8 nM by serial dilution in PBS/Tween. The six samples (150 µl) were injected for 90 sec at a flow rate of 100 µl/min.

Detailed Kinetic and Equilibrium Analysis

In the second injection cycle, the interaction of TEM1 with BLIP-F142A was investigated. TEM1 was immobilized to five different levels in five channels at pH 4.0, which was found to be optimal in the first part of the study (see Results). The immobilization level of TEM1 in each channel was controlled by activating the ligand channel surfaces of a second ProteOn GLC sensor chip using various concentrations of EDAC and sulfo-NHS as follows: channels 1 and 2, 100 mM EDAC and 25 mM sulfo-NHS; channels 3 and 4, 50 mM EDAC and 12.5 mM sulfo-NHS; channel 5, 25 mM EDAC and 6.3 mM sulfo-NHS. The sixth channel was again left unmodified to serve as a reference channel. Five samples of TEM1 protein (2 µM) were prepared in 10 mM ProteOn acetate buffer, pH 4.0, and these five samples (180 µl) were injected at a flow rate of 30 µl/min. To deactivate remaining surface groups, 1 M ethanolamine HCl, pH 8.5 was then injected into the five activated channels. A sample of running buffer was included in each step for injection into the reference channel.

Immobilization was followed by injection of the BLIP-F142A concentration series. Six BLIP-F142A samples (240 µl, same concentrations as above) were injected for 6 min into the six analyte channels at a flow rate of 40 µl/min. The slower flow rate and longer injection time were used to allow the interaction to approach equilibrium.

Sensorgram Analysis

The sensorgrams were processed for baseline alignment and reference channel subtraction. Kinetic analysis was performed by globally fitting curves describing a simple 1:1 bimolecular reaction model to a set of six sensorgrams.

Results

Optimization of Ligand Immobilization Conditions

The TEM1 ligand density for each pH immobilization condition was determined from the average SPR response of the six interaction spots along each ligand channel (Table 1, sensorgrams not shown). Maximum ligand immobilization (742 RU) occurred at pH 4.0. Ligand immobilization was negligible at pH 5.0 due in part to loss of net charge on TEM1 at its isoelectric point (pI). Immobilization was also reduced at pH 3.0 due to partial loss of charge on the surface near the ionization constant (pK_a) of the functional surface groups. Loss of net charge on either the tested protein or chip surface greatly decreases the electrostatic preconcentration of protein to the surface that is needed for efficient immobilization.

Table 1. pH dependence of TEM1 immobilization. TEM1 protein was immobilized in ProteOn acetate buffer, pH 3.0–5.0. Ligand density was determined from the average SPR response of the six interaction spots along each ligand channel.

Channel	Buffer pH	Ligand Density (RU)
1	3.0	610 ± 6
2	3.5	681 ± 6
3	4.0	742 ± 17
4	4.5	727 ± 5
5	5.0	167 ± 4

Optimization of Analyte Binding Conditions

The 36 sensorgrams generated in the first TEM1/BLIP-142A injection cycle were grouped into six sets of six, with five sets corresponding to the interaction of the BLIP-F142A concentration series with each immobilization condition (Figure 1).

Kinetic rate constants were calculated for the four immobilization conditions that yielded measurable responses (pH 3.0–4.5). These constants were similar, with association rate constants (k_a) of 6.7–7.9 x 10^4 $M^{-1}sec^{-1}$, dissociation rate constants (k_d) of 1.56–1.74 x 10^{-2} sec^{-1}, and equilibrium dissociation constants (K_D) of 200–250 nM.

Ligand activity and maximum analyte binding differed among these four immobilization conditions. The maximum response of 600 nM BLIP-F142A occurred upon interaction with TEM1 immobilized at pH 4.0, the pH value corresponding to the highest TEM1 ligand density and highest ligand activity ($\%R_{max}$) achieved (Figure 2).

Ligand activity expresses the percentage of immobilized TEM1 capable of binding BLIP-142A. TEM1 ligand activity was calculated as $\%R_{max}$, the ratio of the measured R_{max} of 300 nM BLIP-F142A with the expected R_{max}. The expected R_{max} was determined by multiplying the TEM1 ligand density by the ratio of the molecular weights of BLIP-142A and TEM1 (assuming 1:1 binding); this value represents the level of response expected if 100% of the TEM1 molecules were to bind BLIP-142A. The maximum ligand activity observed here was in the range of 20% (Figure 2), a level sufficient for kinetic analysis and one that can be difficult to attain for many classes of proteins.

The dependence of TEM1 ligand density and activity, and the BLIP-F142A analyte response on the immobilization conditions (buffer pH) follow bell-shaped curves. That each of these parameters attains its optimal value when TEM1 is immobilized at pH 4.0 indicates the protein is preconcentrated most efficiently at this pH, and also maintains maximum activity and binding capacity.

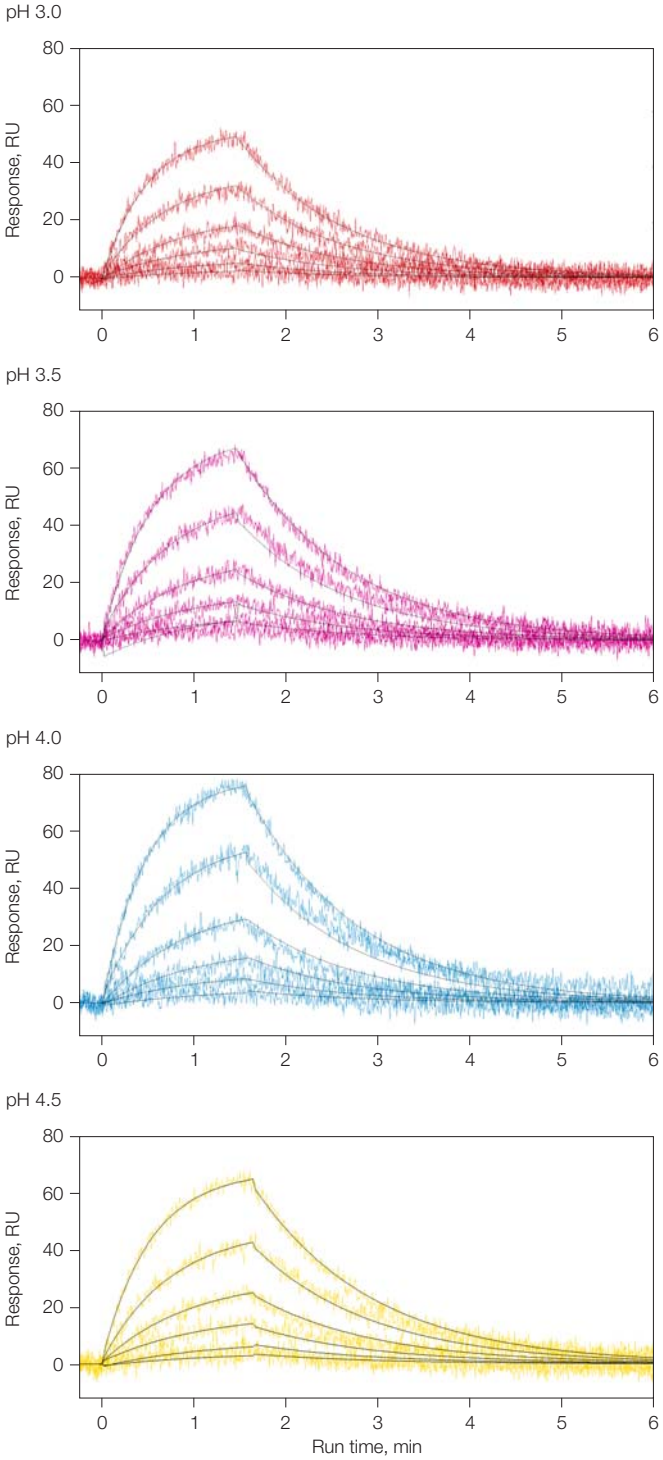

Fig. 1. Kinetic analysis of the TEM1/BLIP-F142A interaction for determination of optimal interaction conditions. Sensorgrams are shown for the four conditions tested that yielded measurable responses. BLIP-F142A concentrations in all panels are 600, 300, 150, 75, 37.5, and 18.8 nM (from top to bottom). Black lines represent the global fit of the sensorgrams to a 1:1 kinetic interaction model.

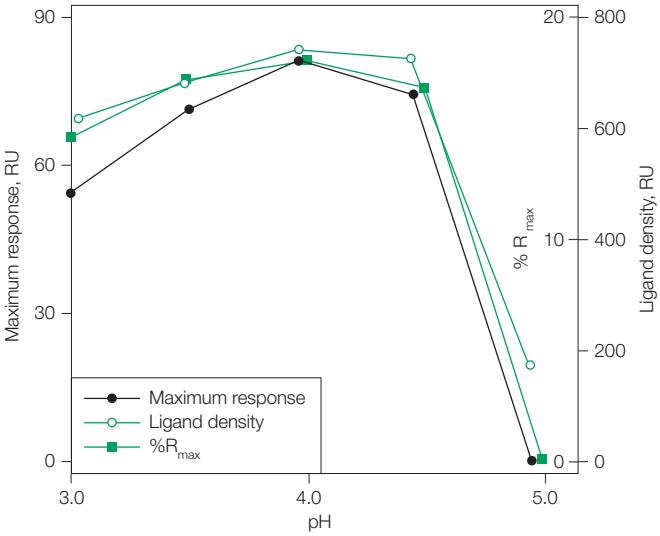

Fig. 2. The dependence of the BLIP-F142A analyte response, TEM1 ligand density, and TEM1 ligand activity (%R_{max}) on the pH of the immobilization buffer. Scales adjusted to align the response of each parameter to the same plot.

Kinetic and Equilibrium Analyses

The 36 sensorgrams generated in the second injection cycle were also grouped into six sets of six, with five of the sets corresponding to the interaction of a BLIP-F142A concentration series with each TEM1 immobilization level (Figures 3 and 4) and the sixth to the reference channel. Equilibrium dissociation constants (K_D) were obtained from these sensorgrams by both kinetic and equilibrium analysis methods.

For kinetic analysis, each set of six sensorgrams was globally fitted to a 1:1 bimolecular kinetic model (Figure 3) to obtain the association rate constant (k_a) and dissociation constant (k_d); K_D is given by the ratio k_d/k_a. For equilibrium analysis, the same sensorgrams were used, and the response at equilibrium of the interaction was measured in each sensorgram for each of the six analyte concentrations. These equilibrium response levels (R_{eq}) were then fitted to a simple bimolecular equilibrium model (Figure 4), from which K_D was derived as that concentration producing a response equal to 50% of the response at saturation.

The kinetic rate constants and equilibrium dissociation constants obtained for all experiments by both analysis modes are shown in Table 2. Note that similar equilibrium dissociation constants were obtained by both modes of analysis.

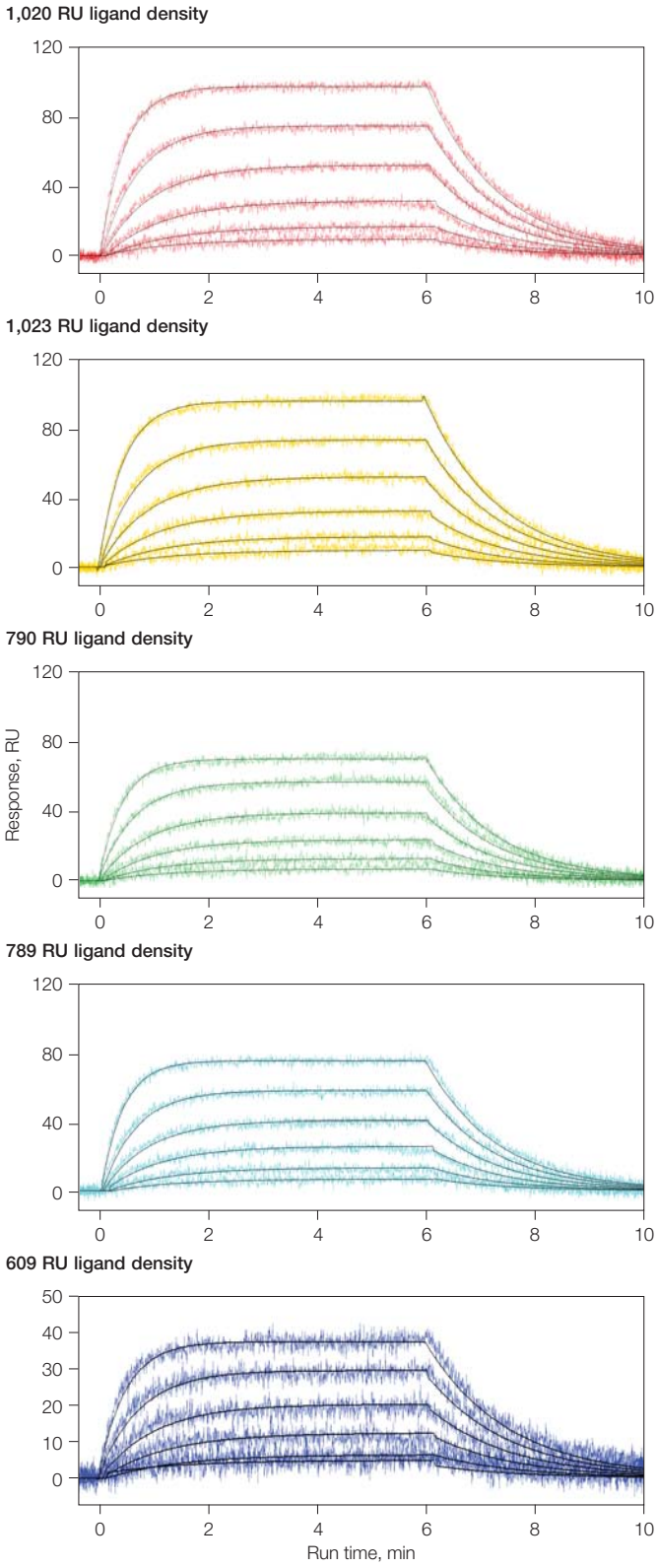

Fig. 3. Kinetic analysis of the TEM1/BLIP-F142A interaction for determination of kinetic rate constants k_d and k_a (at pH 4.0). Sensorgrams are shown for the five levels of TEM1 immobilization. BLIP-F142A concentrations are 600, 300, 150, 75, 37.5, and 18.8 nM (traces from top to bottom). Black lines represent the global fit of the sensorgrams to a 1:1 kinetic interaction model. See Table 2 for the kinetic rate and equilibrium dissociation constants derived from these data.

Table 2. Summary of TEM1/BLIP-F142A kinetic rate constants. TEM1 was immobilized at pH 4.0. The equilibrium dissociation constant value determined by kinetic analysis was calculated from k_d/k_a.

Ligand Density	Kinetic Analysis			Equilibrium Analysis
(RU)	k_a ($M^{-1}sec^{-1}$)	k_d (sec^{-1})	K_D (nM)	K_D (nM)
1,020	8.55×10^4	1.27×10^{-2}	148	140
1,023	8.06×10^4	1.25×10^{-2}	155	131
790	8.75×10^4	1.30×10^{-2}	148	126
789	9.41×10^4	1.30×10^{-2}	138	135
609	7.90×10^4	1.12×10^{-2}	141	143
Average	8.53×10^4	1.25×10^{-2}	146	135
Standard error	6.00×10^3	7.46×10^{-2}	6.6	6.9
CV (%)	7.03	5.98	4.5	5.1

Discussion

Determining the optimal immobilization conditions for different proteins can often be tedious and time-consuming. When embarking on an investigation of a protein-protein interaction, one needs to first optimize various parameters of ligand immobilization, including ligand and analyte concentrations, injection times, flow rates, and immobilization buffer characteristics. The optimal pH for the immobilization buffer balances the electrostatic attraction of the protein to the chip surface while minimizing protein deactivation. In this study, the optimal pH for immobilization was determined to be that pH that yielded the highest ligand density while maintaining optimal ligand activity and maximum analyte response.

Testing a range of ligand immobilization and analyte binding conditions is essential, even when the pI of the ligand is known. The optimal pH can often be predicted to be about 1 pH unit below the pI, as was found here. However, many factors influence immobilization level and binding response, including protein degradation, changes in protein configuration, and even mutation of a single residue, which can significantly alter a protein's pI.

The capability of the ProteOn XPR36 protein interaction array system to assess multiple immobilization conditions minimizes the time and resources needed to achieve optimal results. Sensor chip usage is minimized by simultaneous measurement of 36 interactions on a single chip. Reagent consumption can be kept low by using a short injection time during preliminary screening, and time is saved by seeing the results of testing in parallel.

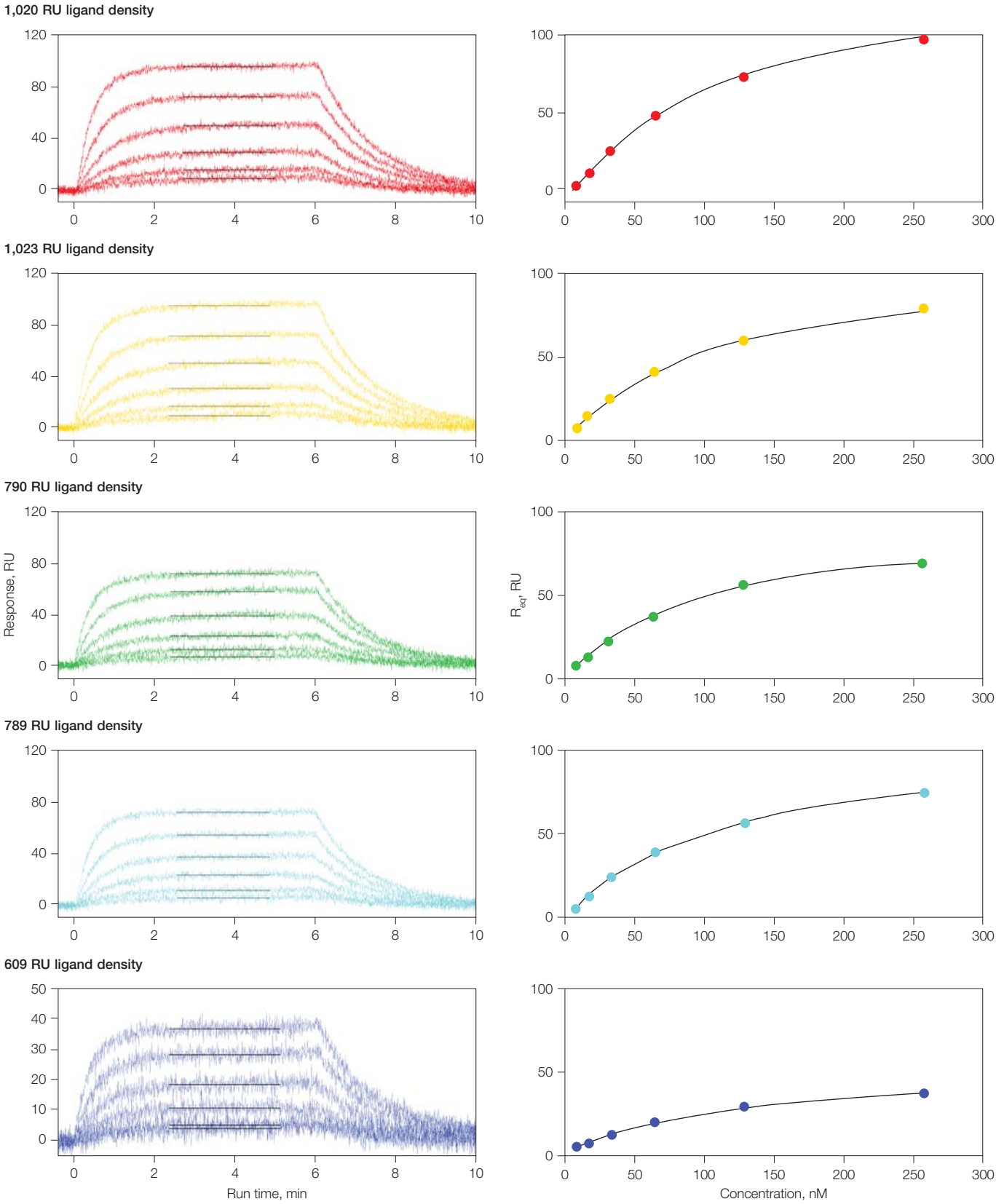

Fig. 4. Equilibrium analysis of the TEM1/BLIP-F142A interaction for determination of the equilibrium dissociation constant, K_D. Left panels, sensorgrams for the five levels of TEM1 immobilization. BLIP-F142A concentrations in all panels are 600, 300, 150, 75, 37.5, and 18.8 nM (traces from top to bottom). Black lines represent the equilibrium response level (R_{eq}) for each analyte concentration. Right panels, plots of R_{eq} as a function of concentration, with the curves fit to a 1:1 equilibrium model for determination of K_D at 50% saturation response. See Table 2 for the equilibrium dissociation constant values derived from these data.

Conclusions

In this study, the ProteOn XPR36 6 x 6 interaction array was applied both to rapidly optimize binding conditions and to perform a detailed kinetic and equilibrium analysis of the TEM1/BLIP-F142A interaction. Optimization and analysis were each accomplished in a single ligand-analyte injection cycle, demonstrating the power of the ProteOn XPR36 system.

References

Albeck S and Schreiber G, Biophysical characterization of the interaction of the β-lactamase TEM-1 with its protein inhibitor BLIP, Biochemistry 38, 11–21 (1999)

Bronner V et al., Rapid and efficient determination of kinetic rate constants using the ProteOn XPR36 protein interaction array system, Bio-Rad bulletin 3172 (2006)

Bush K, Classification of β-lactamases: Groups 1, 2a, 2b, and 2b', Antimicrob Agents Chemother, 33, 264–270 (1989)

This tech note was current as of the date of writing (2005) and not necessarily the date this version (rev A, 2006) was published.

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *800 4BIORAD* ***Australia*** *02 9914 2800* ***Austria*** *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 21 3237 9400* ***Canada*** *905 712 2771* ***China*** *86 21 6426 0808* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00* ***France*** *01 47 95 69 65* ***Germany*** *089 318 84 0* ***Greece*** *30 210 777 4396* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 455 8800* ***India*** *91 124 4029300/5013478* ***Israel*** *03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *03 5811 6270* ***Korea*** *82 2 3473 4460* ***Mexico*** *55 5200 05 20* ***The Netherlands*** *0318 540666* ***New Zealand*** *64 9415 2280* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 095 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *27 0861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *061 717 95 55* ***Taiwan*** *886 2 2578 7189/2578 7241* ***United Kingdom*** *020 8328 2000*

Bulletin 5367 US/EG Rev A *05-0808 0606 Sig 1205*