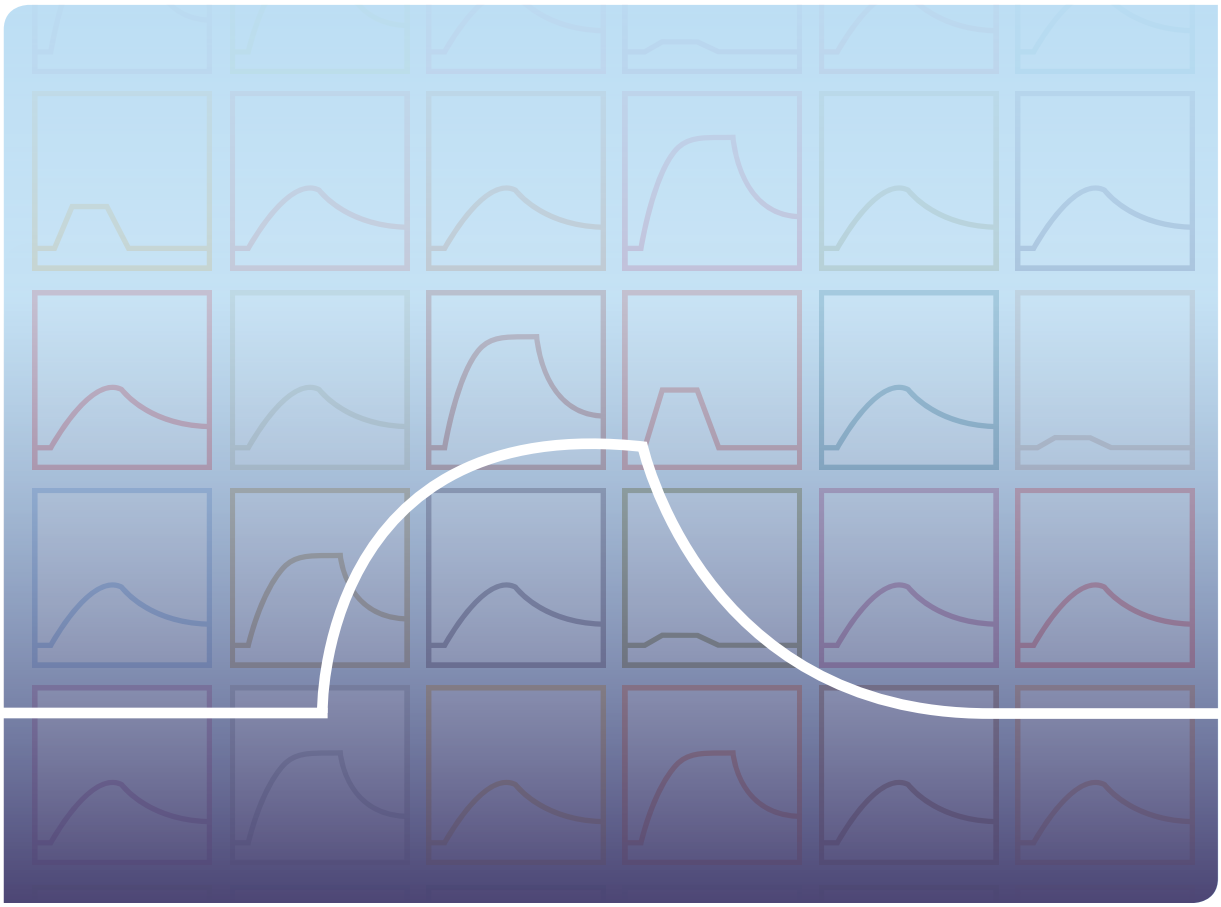
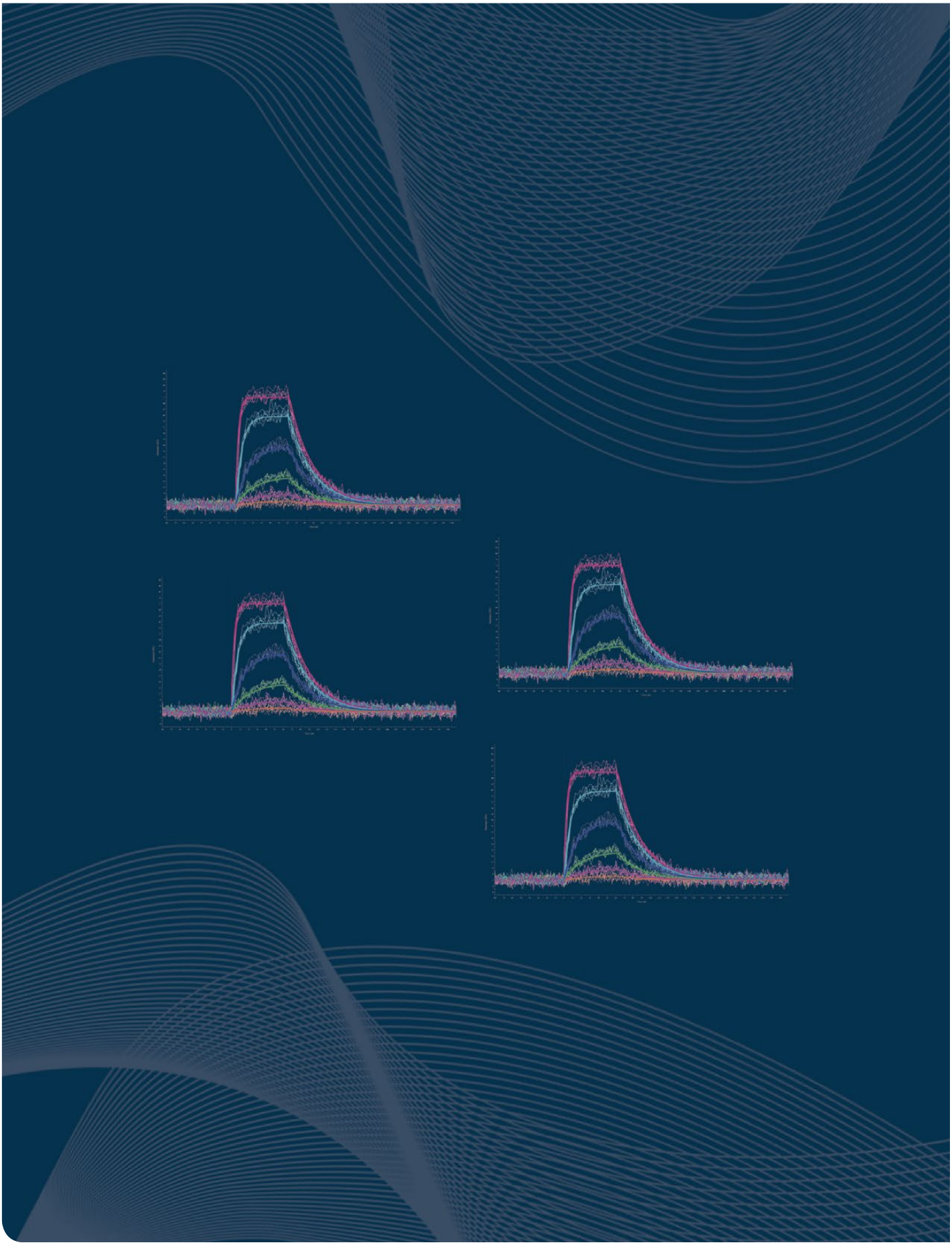
Protein Interaction Analysis



ProteOn™ XPR36 Experimental Design and Application Guide

BIO-RAD



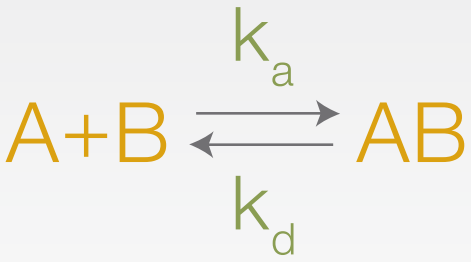
Introduction

Explore the World of Parallel Surface Plasmon Resonance Analysis

Measuring Biomolecular Interactions with SPR

Surface plasmon resonance, or SPR, is a biosensor technology enabling label-free and real-time measurement of biomolecular interactions. A typical SPR experiment involves first immobilizing a ligand (a biomolecule such as a protein or a nucleic acid) to the functionalized surface of a sensor chip and then flowing an analyte (an interacting biomolecule such as another protein or a small molecule) over the chip surface to investigate the binding affinity and binding kinetics between the analyte and the ligand.

The binding of the analyte to the ligand is tracked in real time by following the change in SPR signal over time, and this time-traced graph is called a sensorgram. Fitting the sensorgram to a suitable kinetic binding model allows for the calculation of kinetic parameters such as the association rate constant (k_a in the unit of $M^{-1}s^{-1}$), the dissociation rate constant (k_d in the unit of s^{-1}), and the equilibrium constant (K_D in the unit of M).



Biomolecular interaction in a new light. Two biomolecules, **A** and **B**, interact with each other to form a complex **AB**. Using an SPR biosensor, along with the equilibrium constant $\mathbf{K_D}$, the association rate constant $\mathbf{k_a}$ and the dissociation rate constant $\mathbf{k_d}$ can be measured, determining more details of the interaction compared to other methods.

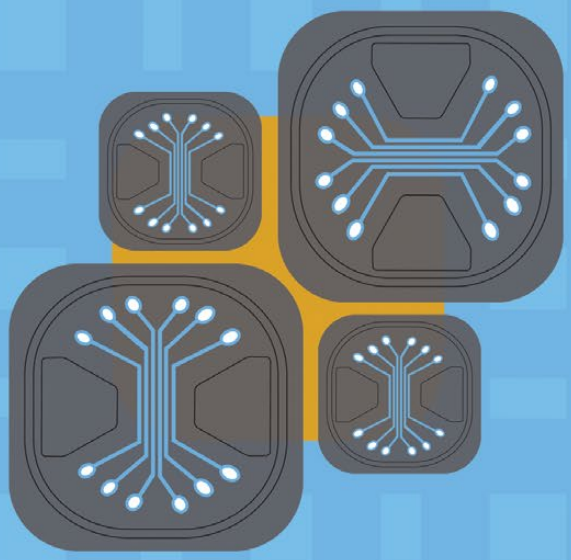
Advantages of the ProteOn™ XPR36 System

The ProteOn XPR36 protein interaction array system is an SPR biosensor platform that provides real-time label-free analysis of the specificity, affinity, and kinetics of biomolecular interactions. Using the XPR36 configuration, the system features a novel 6 x 6 interaction array for the simultaneous analysis of up to six ligands with up to six analytes. The unique design increases the versatility of experimental design and the productivity of experimental workflow, enabling the completion of high quality SPR experiments with high efficiency. The advantages position the ProteOn XPR36 system as an optimal SPR platform in label-free drug discovery and structural biology applications with high quality results and excellent cost-effectiveness.

The parallel-flow SPR biosensor platform:

- Analyzes up to 36 different protein interactions in a single run on a single chip
- Measures a variety of experimental conditions simultaneously using parallel-flow fluidics
- Screens multiple panels of analytes
- Acquires the resonance angle shift as SPR response units (RU) for accurate kinetics
- Employs One-shot Kinetics™ technology, which enables a complete kinetic analysis in a single run

This book describes how to apply the ProteOn XPR36 system for SPR analysis of biomolecular interactions. It includes technical introductions, user guides, and tips and techniques.



CHAPTER 1
ProteOn™ XPR36 Technology

Bio-Rad Laboratories has remained at the center of scientific discovery for more than 60 years. The company is renowned worldwide among hospitals, universities, and major research institutions, as well as biotechnology and pharmaceutical companies, for its commitment to quality and customer service. Among Bio-Rad's groundbreaking contributions is the ProteOn XPR36 protein interaction array system, an optical biosensor capable of simultaneously analyzing 36 individual biomolecular interactions in a label-free manner. It has been widely accepted and used as a fully automated, high-performance, and high-throughput surface plasmon resonance (SPR) biosensor platform.

1.1 ProteOn XPR36 Technology Overview

Surface plasmon resonance (SPR) is an optical phenomenon that occurs when p-polarized light at a certain wavelength and angle is reflected off a thin metal film (the gold film coated on a sensor chip) under the condition of total internal reflection (TIR). The light excites surface plasmons in the metal at a certain incident angle. The TIR field generates an evanescent wave in the thin metal film that extends hundreds of nanometers from the surface into the medium above, in this case the molecules in contact with the chip surface. The excited surface plasmons are very sensitive to the refractive index change at the surface of the thin metal film. Thus the incident angle of the light required for SPR is impacted by the refractive index change of the molecules in contact with the chip surface. In an SPR binding experiment, this refractive index change is brought about by binding of analyte in solution to ligand immobilized on the chip surface; therefore, tracking the change in the incident angle required for SPR allows one to monitor biomolecular interactions in real time. The change of the incident angle required for SPR is defined as SPR response in the unit of response unit (RU). 1 RU is 1/1,000,000 of 1 refractive index unit, and is roughly equivalent to a surface density of protein at approximately 1 pg/mm^2. For a more in-depth discussion of SPR, see a recent review that offers an overview of SPR theory and different SPR configurations (Daghestani et al. 2010). This reference describes the theory and application of a number of optical biosensors, including surface plasmon resonance biosensors, and the different configurations for each.

Plotting the SPR response over time during the interaction between an analyte and a ligand results in a sensorgram. A sensorgram is a visual presentation of the interaction. Figure 1.1 illustrates the terms for a sensorgram using, for instance, an antibody-antigen interaction. The binding response initially increases as analyte is flowed over the sensor chip and associates with the immobilized ligand and then decreases as the analyte solution is replaced with buffer and the binding complex dissociates. If binding equilibrium is reached during the association phase, the sensorgram will reach a constant plateau before the analyte solution is replaced with buffer and the binding complex dissociates. Fitting the sensorgram data to a binding model allows for the calculation of the association and dissociation rate constants and determination of the binding affinity. Traditionally, kinetic measurements with SPR usually involve sequential injections of analyte at increasing concentrations over the same ligand surface, which requires complete removal of the analyte or regeneration of the ligand surface between analyte injections. In an ideal case, regeneration of the ligand surface is observed in the sensorgram as a sharp response change after dissociation to restore the baseline to the original level. Regeneration is usually done with a combination of dilute surfactants, salts, and acids or bases; however, care must be taken during regeneration to avoid denaturing the immobilized ligand or removing ligand from the sensor chip.

The ProteOn XPR36 system characterizes the following aspects of a biomolecular interaction:

- Specificity of the interaction
- Rate of the interaction ($\mathbf{k_a}$)
- Stability of the complex ($\mathbf{k_d}$)
- Strength of the interaction ($\mathbf{K_D}$ [$\mathbf{K_D = k_d / k_a}$])

The parameters are obtained from the data fitting of the association, equilibrium (optional), and dissociation phases of a sensorgram.

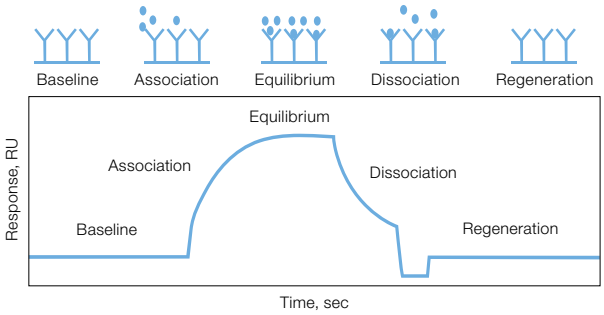


Fig. 1.1. SPR sensorgram. Surface (—); ligand (Y); analyte (●). RU, response units.

The ProteOn XPR36 system offers a distinct advantage over other SPR biosensor platforms because the unique 6 x 6 interaction array of the ProteOn sensor chips enables the One-shot Kinetics™ approach measuring the interaction of one ligand with a six-concentration series of one analyte in a single injection. This approach eliminates the need for traditional regeneration of the sensor chip between analyte injections, which often deteriorates the ligand surface. Using enhanced microfluidic delivery and XPR™ technology, the ProteOn XPR36 system can immobilize up to six separate ligands on a single sensor chip in six separate flow cells and then rotate the sensor chip 90 degrees to flow up to six separate analytes over the ligand surfaces (Figure 1.2).

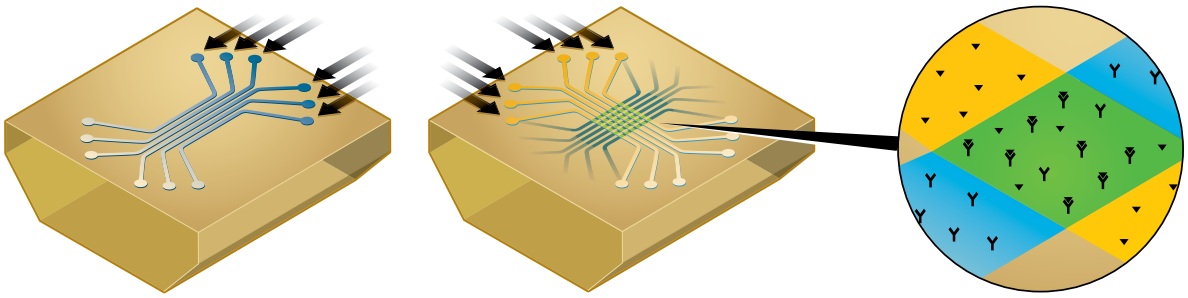
This unique feature of the ProteOn allows for the detection of up to 36 separate interactions on a single sensor chip and significantly increases the throughput of SPR biosensing. In a recent study, the ProteOn XPR36 system was used to immobilize 36 different ligands in a stepwise immobilization procedure designed for the high-throughput epitope mapping and binding of antibody-antigen interactions (Abdiche et al. 2011). The 6 x 6 interaction array of the ProteOn sensor chips also allows for inline referencing, whereby data from unmodified spots in between the immobilized ligand spots on the sensor chip are used to subtract out artifacts such as noise and baseline drift. This inline referencing is superior to referencing with a separate flow cell and means the ProteOn XPR36 system can collect high-quality SPR data at the low signal-to-noise ratios often seen with small molecule analytes.

1.2 What Kind of Information is Obtained with the ProteOn XPR36 System?

As an SPR biosensor platform, the ProteOn XPR36 system provides a wide variety of important information on biomolecular interactions such as the specificity, affinity, qualitative ranking, kinetics, and thermodynamics of binding.

The ProteOn XPR36 system can be used in pharmaceutical drug discovery, antibody characterization, immunogenicity testing, the development and manufacture of biologics, or for clinical research. It could also be used in other fields where there is a need for label-free characterization of biomolecular interactions. Key applications include:

- Quantification of binding affinity and kinetics
- Determination of binding specificity and the number of binding sites
- Characterization of the mechanism of action
- Confirmation of biomolecule binding to a target
- Screening of fragment libraries
- Validation of IC_{50}/EC_{50} values during hit-to-lead optimization
- Characterization of immune responses



Step 1
Immobilize up to 6 ligands in the vertical direction.

Step 2
Inject up to 6 analytes in the horizontal direction.

Step 3
Detail showing one of 36 interaction spots.

Fig. 1.2. The ProteOn 6 x 6 interaction array on a sensor chip.

Some classic applications of the ProteOn XPR36 system are in antibody engineering. Epitopes on an antigen can be characterized by epitope mapping, a process by which the affinities of an antibody to site-directed mutants of a single antigen help pinpoint the location of an epitope. An investigation of the epitope specificity, or epitope binding, of different antibodies can be done on the ProteOn XPR36 system using the sandwich assay. In this assay, a second antibody is injected over a previously-formed antigen-antibody complex to see whether or not the second antibody can still bind. Binding of the second antibody to the antigen-antibody complex, or the formation of a "sandwich" is an indication that the second antibody recognizes a different epitope than the first antibody.

SPR can also be used to determine the active concentration of an analyte in a crude or impure sample by probing the sample of interest under mass transport control using a low flow rate and/or a high-capacity sensor chip bearing an analyte-specific ligand. Under such mass transport limited conditions the association rate of binding, or the initial binding rate, is proportional to the concentration of analyte in solution. The concentration of analyte in a crude sample can be calculated by comparing the initial binding rate to a standard curve of initial binding rates for known concentrations.

1.3 How are Kinetic Parameters Obtained?

By fitting sensorgram data from a ProteOn XPR36 system experiment to a suitable binding model, kinetic parameters such as the association rate constant (k_a), dissociation rate constant (k_d), and the equilibrium or affinity constant (K_D) can be extracted. Kinetic data are crucial for characterizing an interaction, as they allow for a thorough understanding of the nuances of binding. Interactions with the same affinity can have markedly different association and dissociation rates, as seen in Figure 1.3. An antibody or small molecule that has a high affinity (low K_D value) for a protein target may be a poor drug in vivo if it has a very high dissociation rate and thus can be easily displaced by another molecule. This kind of information is easily obtained from an SPR experiment but would not be uncovered using a method such as isothermal calorimetry (ITC) that measures binding affinity based on binding at equilibrium. In addition, knowing the kinetics of a small molecule interaction allows for more accurate analysis of quantitative-structure activity relationships, as different structures can be evaluated by their separate effects on association and dissociation as opposed to affinity alone.

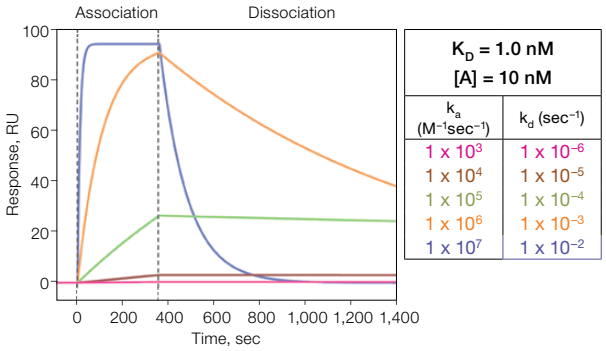


Fig. 1.3. Sensorgram plots showing the response (in RU) versus time for five different interactions with the same affinity (K_D = 1.0 nM) but markedly different association (k_a) and dissociation (k_d) rate constants. A, antibody.

Figure 1.4. The experimental workflow of the 6 x 6 interaction array.

ProteOn Manager™ software gives you the option of using seven different binding models to analyze your sensorgram data:

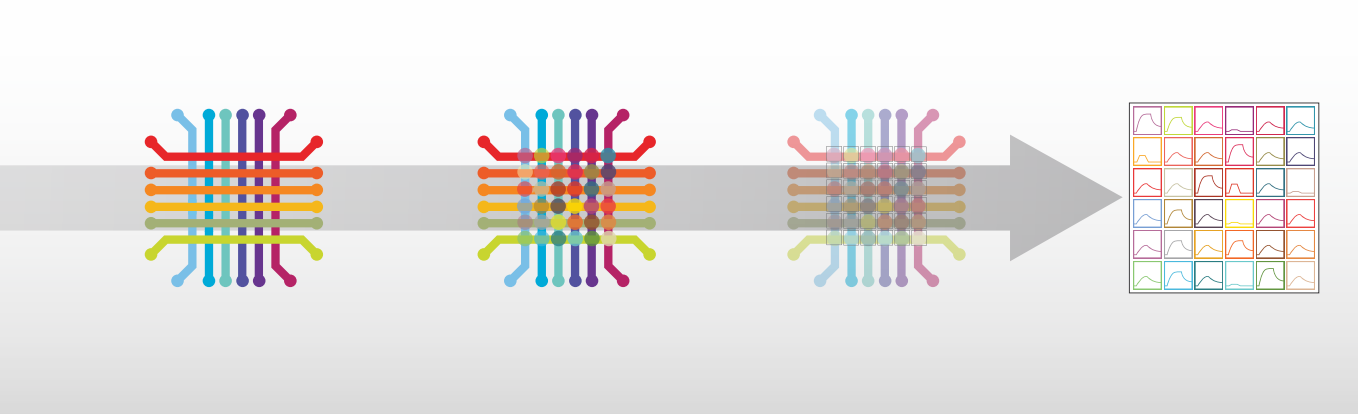
- **Langmuir** — simple 1:1 bimolecular interaction
  - Simultaneous fitting of k_a and k_d
  - Fitting of k_d only
- **Langmuir with drift** — simple 1:1 biomolecular interaction with a constant baseline drift taken into account
- **Langmuir with mass transport limitations** — simple 1:1 biomolecular interaction that takes into account the rate of diffusion of analyte from the bulk to the chip surface
- **Bivalent analyte** — one analyte has two binding sites to one ligand
- **Heterogeneous analyte** — two analytes compete for binding to one ligand
- **Heterogeneous ligand** — one analyte binds to two ligands
- **Two-state conformation** — accounts for a change in conformation of the binding complex that occurs after the analyte binds. In addition, it is possible to calculate the affinity value (K_D) using equilibrium analysis, in which the equilibrium responses at different analyte concentrations are filled to a simple saturation binding model

1.4 Advantages of the 6 x 6 Interaction Array

When running an experiment using the ProteOn XPR36 system, the 6 x 6 interaction is formed. The experimental workflow is shown in Figure 1.4.

The 6 x 6 interaction array brings these benefits to SPR experiments:

- Experimental versatility
- High productivity
- High data quality



Experimental Versatility — Multiple Experimental Configurations and Fast Qualitative and Quantitative Assays

Kinetic Characterization (1-to-1)

In kinetic characterization experiments, the optimization of experimental protocols is usually the most labor-intensive and time-consuming step. Probing at one time six ligand immobilization conditions together with six analyte injection conditions, the ProteOn XPR36 system allows for full optimization in a single run. This ensures the optimal experimental conditions for the interaction between the ligand and the analyte. The method of using a single run of 6 x 6 injections for a complete kinetic analysis is called the One-shot Kinetics approach.

6 variations of a target

6 concentrations of the same analyte

Kinetic Screening (6-to-1)

In kinetic screening experiments, each of the six ligand channels gives a full kinetic analysis in a single run. This high throughput enables fast processing of a large number of samples while accurate kinetics is maintained. The ProteOn XPR36 system provides the best balance between throughput and accuracy of kinetic screening.

6 different targets

6 concentrations of the same analyte

Multiplex Screening (6-to-6) and Array Screening (36-to-1)

In multiplex or array screening experiments, the 6 x 6 interaction array of the ProteOn XPR36 system is fully utilized for high throughput, which enables multiplex or 36-ligand screening.

6 different targets

6 different analytes

36 different targets

1 analyte

High Productivity — Fast Protocol Optimization and High Throughput

Sensor chip surface

Kinetic Characterization

- ProteOn XPR36 system: 1.1 hr, 6 full kinetics (36 data points, surface regeneration not required)
- Conventional serial flow SPR system: 3.5 hr, 3 full kinetics (18 data points, surface regeneration required)

Kinetic Screening

Captured Ligand Screening (for mAb supernatants)

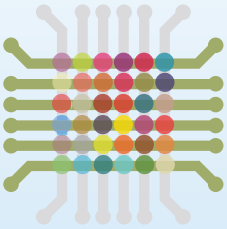
- ProteOn XPR36 system: 11 hr, 96 full kinetics (576 data points)
- Conventional serial flow SPR system: 65 hr, 96 full kinetics (576 data points)

Analyte Screening

- ProteOn XPR36 system: 25 hr, 96 x 6 full kinetics (3,456 data points)
- Conventional serial flow SPR system: 5.7 days, 96 x 3 full kinetics (1,728 data points)

Multiplex Screening

- ProteOn XPR36 system: 0.7 hr, 6 x 6 binding matrix (36 data points)
- Conventional serial flow SPR system: 2.5 hr, 3 x 6 binding matrix (18 data points)



Array Screening

- ProteOn XPR36 system: 12 hr, 36 x 36 binding matrix (1,296 data points)
- Conventional serial flow SPR system: no equivalent

High Data Quality — Four Factors of the ProteOn XPR36 System for High-Quality SPR Results

1 SPR System

Sufficient Signal-to-Noise Ratio

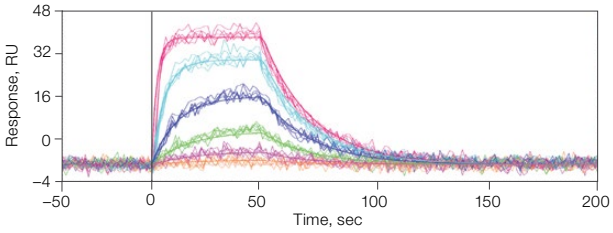


Fig. 1.5. ProteOn XPR36 system signal-to-noise ratio. ProteOn XPR36 system noise is 1 RU and ~2 RU after double referencing. SPR responses over three times signal-to-noise ratio (3 x SNR) are detectable. RU, response units.

Instrument Stability

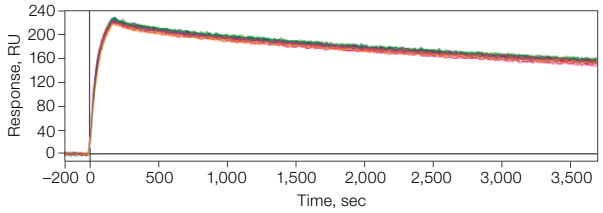


Fig. 1.6. Evaluation of kd value reproducibility using the ProteOn One-shot Kinetics kit. 2 systems x 3 chips x 6 ligand channels x 6 analyte channels = 216 sensorgrams. CV = 6.1% (over 2 systems and 6 sensor chips). RU, response units.

2 Experiment Design

ProteOn XPR36 System Configuration Optimizes Multiple Factors Simultaneously

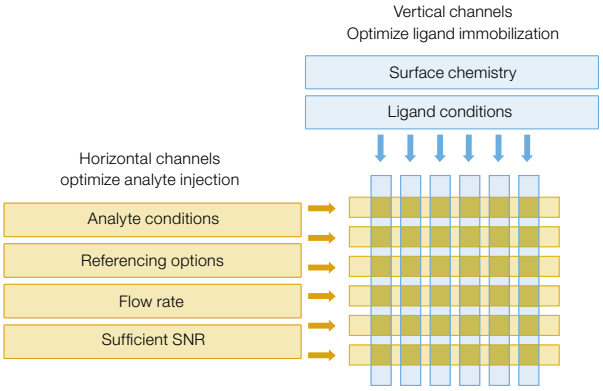


Fig. 1.7. Optimal experimental conditions are obtained in a single run.

3 Data Processing

Data Referencing

The key step in data processing is data referencing. Data referencing corrects for the artifacts in SPR experimental results.

The ProteOn XPR36 system has two novel advantageous referencing modes that no other SPR system provides: an interspot reference to correct for refractive index change (bulk effect) and nonspecific binding, and a real-time injection reference to correct for baseline drift resulting from the changes in the ligand surface.

Note: For additional information about the referencing options in the ProteOn XPR36 system, watch the video at **www.bio-rad.com/proteon/reference**.

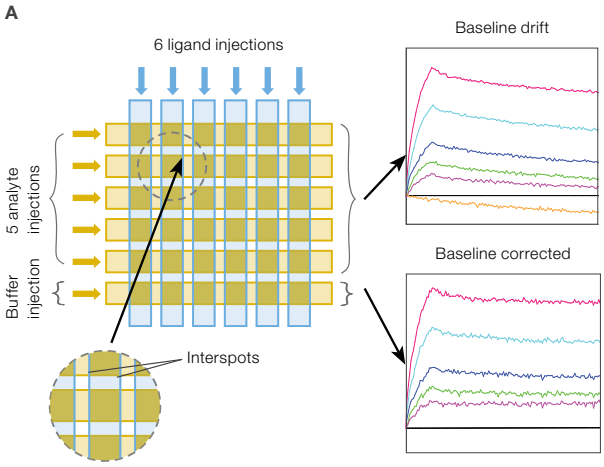


Fig. 1.8. Novel ProteOn XPR36 system references. The ProteOn XPR36 system provides **A**, an interspot blank surface reference to save interaction spots and provide immediate proximate referencing and **B**, a real-time injection reference to correct the exponential baseline drift when using ligand-capture surface chemistry.

4 Data Analysis

Software Advantages

ProteOn Manager software is a comprehensive, user-friendly tool for the analysis of biomolecular interactions. Features include:

- Ease of use
- Integration of data acquisition, data processing, and data analysis
- Powerful graphic user interface
- Intuitive protocol writing interface
- Fast and accurate data processing
- Accurate fitting with 8 models
- Rapid data analysis
- Concise analysis reports
- Export functions for further data processing in Excel or other software

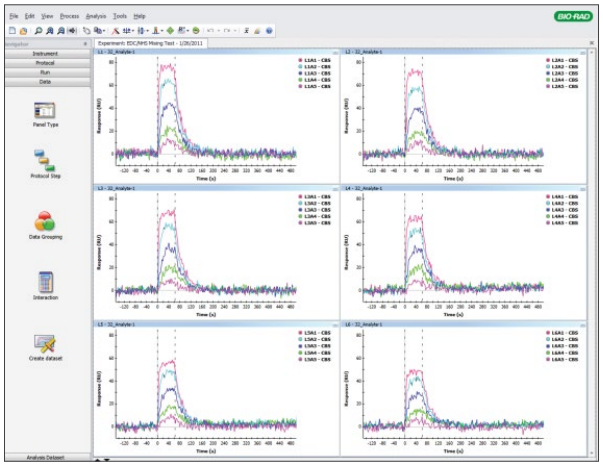


Fig. 1.9. ProteOn Manager software data analysis window.

1.5 ProteOn Webinar Library

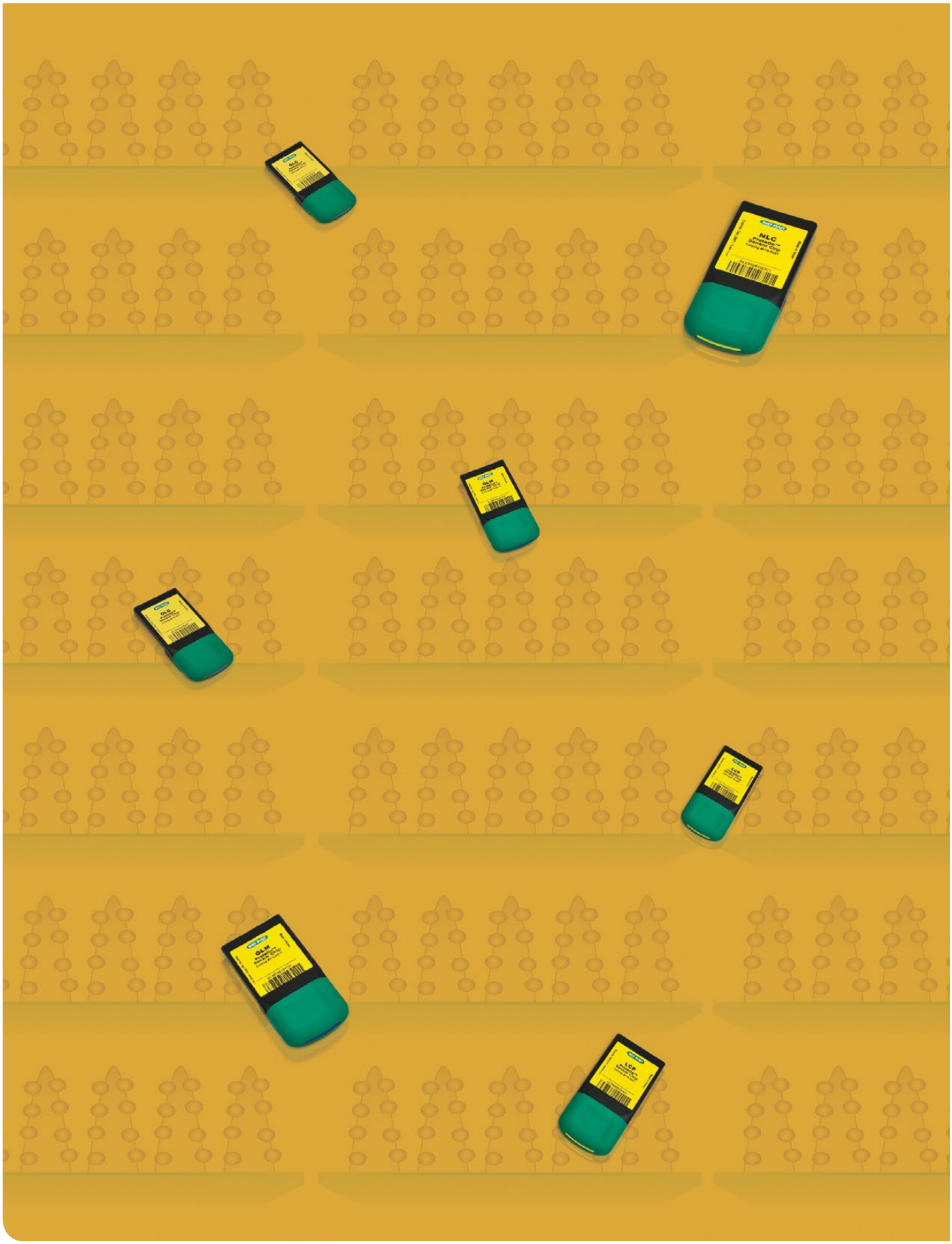
The ProteOn webinars feature presentations from thought leaders in the field of label-free biomolecular interaction analysis who use surface plasmon resonance (SPR) technology.

Visit www.bio-rad.com/info/proteon to view Bio-Rad's extensive library of ProteOn webinars and to sign up for future webinars.

References

Abdiche YN et al. (2011). Expanding the ProteOn XPR36 biosensor into a 36-ligand array expedites protein interaction analysis. Anal Biochem 411, 139–151.

Daghestani HN et al. (2010). Theory and applications of surface plasmon resonance, resonant mirror, resonant waveguide grating, and dual polarization interferometry biosensors. Sensors 10, 9630–9646.



CHAPTER 2
ProteOn™ Sensor Chips

Used with the ProteOn XPR36 system, ProteOn sensor chips are built with an alginate polymer matrix (or a self-assembled monolayer of organic molecules) bound to a thin gold film on a sensor prism. The sensor chips can be functionalized with several different reactive groups to achieve a variety of immobilization surface chemistries. The surface chemistry of the ProteOn sensor chips allows the ProteOn XPR36 system to detect tight binding interactions down to picomolar concentrations of analytes, or analytes as small as 95 daltons (Da). The combination of ProteOn sensor chips with the unique 6 x 6 interaction array allows the interaction analysis of up to 36 separate ligand/analyte pairs on a single chip, thereby increasing the throughput of a single experiment.

2.1 Overview

The ProteOn XPR36 system uses the 6 × 6 interaction array of the ProteOn sensor chips. ProteOn sensor chips contain more than just a gold layer; they are coated with a modified alginate polymer that provides a solution-like, biomimetic environment for ligand immobilization. The general-use ProteOn GLC, GLM, and GLH chips are functionalized with carboxyl groups that react with surface-exposed amines on the ligand, tethering the ligand to the chip surface in a random orientation. The ProteOn NLC chip is coated with NeutrAvidin for immobilization of biotinylated ligands, and the HTG and HTE chips feature a tris-nitrilotriacetate (tris-NTA or 3 x NTA) surface for immobilization of histidine-tagged proteins. The respective chips can be used to capture a ligand at a site-specific location. The ProteOn LCP chip is used with the LCP capturing reagent kit for liposome capture. Although choosing a suitable sensor chip for a particular interaction requires some research and planning, this is time well spent, considering the high-quality data that can be obtained with the right sensor chip.

2.2 ProteOn Sensor Chip Surface Chemistry

ProteOn sensor chips are built with an alginate polymer matrix bound to a thin gold film on a sensor prism. The alginate matrix can be functionalized with several different reactive groups to facilitate different immobilization surface chemistries. The hydrophilic nature of the alginate layer creates a solution-like environment that prevents denaturation of the immobilized ligand and nonspecific adsorption of the analyte. The increased surface area of the 3-D structure of the alginate layer provides more attachment sites than would a completely flat surface and results in more ligand molecules being immobilized on the chip surface. In addition, the molecular weight and structure of the alginate coating can be modified to create sensor chips with different surface capacities. This results in sensitive detection of interactions with minimal surface effects on binding. The surface chemistry of the ProteOn sensor chips allows the ProteOn XPR36 system to detect tight binding interactions down to picomolar concentrations of analytes, or analytes as small as 95 Da. The ProteOn sensor chips, combined with the unique 6 x 6 interaction array, allow for the interaction of up to 36 separate ligand/analyte pairs on a single chip, increasing the throughput of a single experiment.

The ProteOn sensor chips are based on innovative, patent-protected surface chemistry. The general-use ProteOn sensor chips (GLC, GLM, and GLH sensor chips) come functionalized with carboxyl groups to facilitate amine coupling of protein ligands via surface-exposed amine groups. In addition to serving as attachment sites, the carboxyl groups serve to concentrate the ligand at the surface of the sensor chip as the negatively charged carboxyl groups attract proteins rendered positively charged via incubation in an acidic buffer. Activation of carboxyl groups for ligand immobilization is done using carbodiimide chemistry with the reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS). Once activated, the resulting sulfo-NHS esters are highly amine-reactive and react with free amines exposed on the ligand to immobilize it to the sensor chip. Any unreacted carboxyl groups that remain activated during the immobilization step are deactivated with ethanolamine to prevent immobilization of analyte protein during the subsequent interaction step. The chemical structure of the binding layers forms easily-activated carboxyl groups, rendering especially high binding capacity and ligand activity. It results in more active ligand on the surface, thus higher analyte signals and higher assay sensitivity.

Different amounts of ligand may be amine-coupled to the GLC, GLM, and GLH sensor chips by controlling the amount of ligand in solution during the immobilization, by tuning the activation level, or by adjusting the length of time of the immobilization step. The amount of ligand bound is monitored by following the binding response displayed on the sensorgram in real time. However, specific applications may require a surface with a very high or low ligand surface capacity. In such cases GLH and GLC chips that have a very high or low density alginate coating may be used.

For immobilizing targets through a site-specific tag, ProteOn sensor chips such as the NLC, HTG, and HTE chips can be used. The NLC chip is coated with NeutrAvidin for immobilization of biotinylated ligands, and the HTG and HTE chips are coated with a unique and innovative tris-NTA surface for immobilization of histidine-tagged proteins.

Sensor chips come packaged in a sealed pouch with an inert gas, have a shelf life of two years if stored properly at 4°C, and are guaranteed for six months from the date of receipt. Sensor chips are continually monitored for quality and have excellent spot-to-spot reproducibility within the 6 x 6 interaction array.

Read more to learn about the different sensor chip types (Figure 2.1) and which is best for your specific application.

2.3 Types of ProteOn Sensor Chips

There are seven types of ProteOn sensor chips that can be used for a variety of different immobilization strategies and the creation of different capacity surfaces. The GLC, GLM, and GLH sensor chips are designed for general amine coupling of ligands, whereas the NLC sensor chips are designed for site-specific attachment of biotinylated ligands and the HTG and HTE chips for histidine-tagged proteins, respectively. Figure 2.1 shows an overview of the different sensor chip types and the specific applications in which they are used.

Chip Selector	
GLC	Compact capacity amine coupling for protein-protein interactions
GLM	Medium capacity amine coupling for protein-protein and protein–small molecule interactions
GLH	High capacity amine coupling for protein–small molecule interactions
NLC	NeutrAvidin for biotinylated molecule capture
HTG	Compact capacity tris-NTA for histidine-tagged large molecule capture
HTE	High capacity tris-NTA for histidine-tagged small molecule capture
LCP	Used with the LCP capturing reagent kit for liposome capture

Fig. 2.1. An overview of the seven different types of ProteOn sensor chips (GLC, GLM, GLH, NLC, HTG, HTE, and LCP) with the specific application for each chip listed.

2.3.1 Amine Coupling ProteOn Sensor Chips: GLC, GLM, and GLH

Three ProteOn sensor chips are available for amine coupling. The GLC, GLM, and GLH chips provide for compact, medium, and high ligand surface capacities, respectively. All three of these chips are functionalized with easily-activated carboxylic acid groups that can be reacted with the activation reagents EDC and sulfo-NHS to react specifically with free surface amines of proteins. Bio-Rad offers the amine coupling kit for use with the GLC, GLM, and GLH sensor chips.

GLC Sensor Chip — Compact Binding Capacity

The GLC sensor chip is designed with an extremely thin alginate layer for amine coupling of protein ligands at a compact (~6 kRU) surface capacity. The compact structure of the alginate layer helps mitigate mass transport effects that are more often observed with thicker layers of surface coating. This versatile sensor chip is ideal for analyzing protein-protein interactions (Figure 2.2).

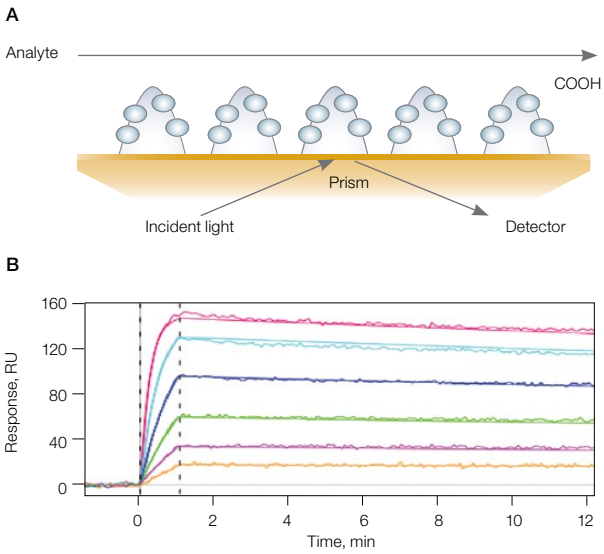


Fig. 2.2. ProteOn GLC sensor chip. A, the thin alginate coating on the GLC sensor chip responsible for creating a compact-capacity surface; **B**, sensorgrams of the interaction between the cytokine IL-2 and the anti–IL-2 antibody using the GLC sensor chip. The IL-2 antibody was immobilized to approximately 2,000 RU, and IL-2 was injected in a twofold dilution series ranging from 80–2.5 nM. The nearly planar surface of the GLC sensor chip allows for high-quality kinetic analysis. RU, response units.

GLM Sensor Chip — Medium Binding Capacity

The GLM sensor chip is coated with a thicker alginate polymer that displays a higher amount of carboxylic acid groups and is thus ideal for creating medium capacity (~12 kRU) ligand surfaces via amine coupling. It can be used for both protein-protein interactions and protein–small molecule interactions (Figure 2.3).

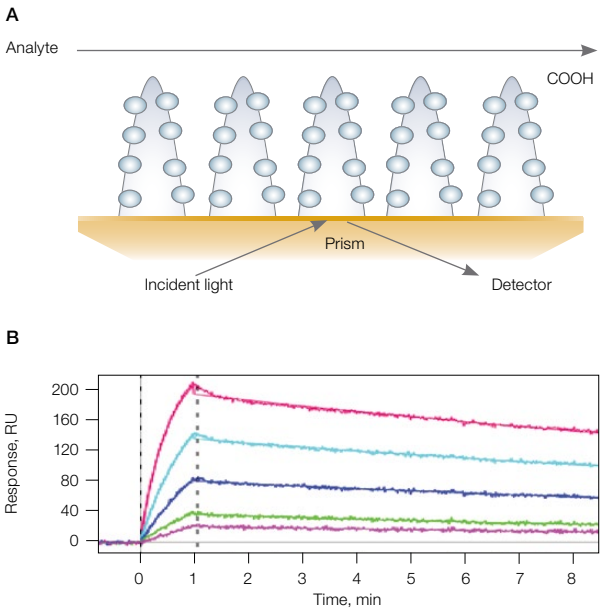


Fig. 2.3. ProteOn GLM sensor chip. A, the extended alginate coating on the GLM sensor chip responsible for creating a medium-capacity surface; **B**, sensorgrams of a TEM1 β-lactamase mutant interacting with the β-lactamase inhibitor protein (BLIP) using the GLM sensor chip. TEM1 was immobilized to approximately 1,500 RU, and BLIP was injected in a twofold dilution series ranging from 600–38 nM. RU, response units.

GLH Sensor Chip — High Binding Capacity

The GLH sensor chip is designed with a high density alginate polymer that contains an increased number of carboxylic acid groups to amine coupling ligands at a very high (>20 kRU) surface capacity. This dense alginate layer on the GLH chip is far superior at binding high capacity ligand surfaces when compared to results of ligand immobilizations done using the GLC and GLM chips (Figure 2.4). This sensor chip is ideal for probing protein–small molecule (<1000 Da) interactions, as the high capacity surface gives an increased binding response. A comparison between the GLH sensor chip and a competitor's high capacity sensor chip shows the full advantage of the ProteOn chip's easily-activated carboxylic groups, rendering significantly higher binding capacity and activity, and thus much higher analyte response (Figure 2.5 and Table 2.1).

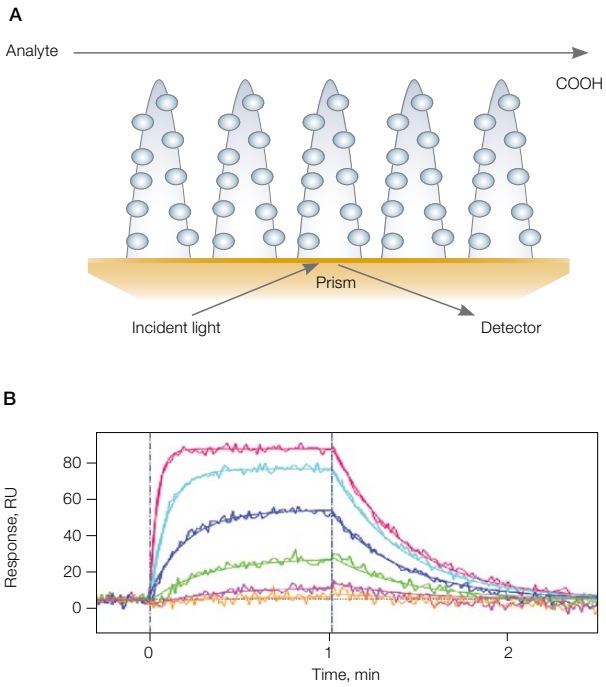


Fig. 2.4. ProteOn GLH sensor chip. A, the dense alginate coating on the GLH sensor chip responsible for creating a high-capacity surface; **B**, sensorgrams of the interaction between the carbonic anhydrase II (30 kD) and the inhibitor 4-carboxybenzenesulfonamide (CBS) (201 Da) using the GLH sensor chip. Carbonic anhydrase II was immobilized to approximately 24,000 RU, and CBS was injected in a threefold dilution series ranging from 20–0.082 µM. RU, response units.

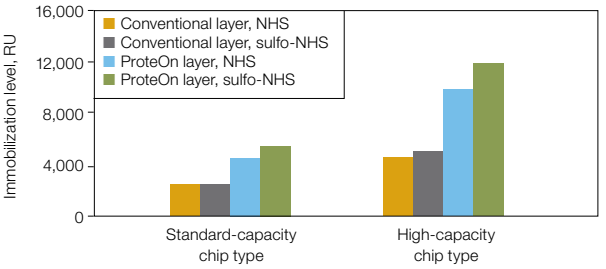


Fig. 2.5. Comparative coupling efficiency. Representative data for immobilization of rabbit IgG. Ligand coupling efficiency of the ProteOn chip's easily-activated layers is higher than in conventional layers, and activation of ProteOn chip layers is higher using sulfo-NHS instead of NHS. RU, response units.

Table 2.1. Representative immobilization efficiencies on ProteOn sensor chip surfaces designed for high protein binding capacity.

Protein	pI	Non-Bio-Rad Chip, NHS Activation, RU	GLM Chip, NHS Activation, RU	GLM Chip, Sulfo-NHS Activation, RU	GLH Chip, Sulfo-NHS Activation, RU
Pepsin	3	70	750	2,050	2,470
Ovalbumin	4.5	2,800	3,400	6,700	6,800
Protein A	5.1	4,300	3,500	6,000	18,800
β2-microglobulin	5.3	2,600	3,250	3,650	12,400
Carbonic anhydrase II	5.9	6,600–2,300	6,000	9,000	21,200
Myoglobin	6.9–7.4	3,900	2,800	7,000	12,200
Polyclonal IgG	6–8	10,000	9,700	12,200	22,200

2.3.2 ProteOn Sensor Chips for Site-Specific Attachment: NLC, HTG, and HTE

NLC Sensor Chip — Immobilization of Biotinylated Ligands

The NLC sensor chip is functionalized with NeutrAvidin bound to the alginate polymer and can capture biotinylated proteins, peptides, and nucleic acids. It can capture ~2,000 RU of IgG or ~500 RU of DNA. The NLC sensor chip is ideal for immobilizing ligands without amine coupling but requires that the ligand be modified with biotin prior to immobilization (Figure 2.6).

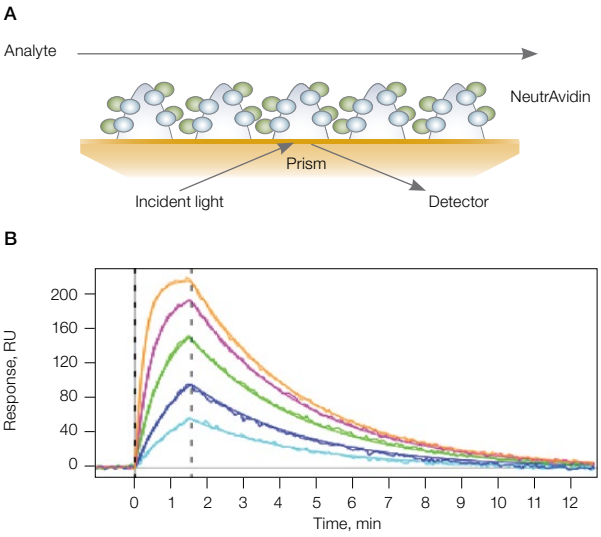


Fig. 2.6. ProteOn NLC sensor chip. A, the NeutrAvidin-modified alginate coating on the NLC sensor chip; **B**, sensorgrams of the interaction between an antibody Fab fragment and biotinylated MHC I/Tyr antigen using the NLC sensor chip. MHC I was captured to approximately 800 RU, and the Fab was injected in a twofold dilution series ranging from 500–31 nM. RU, response units.

HTE and HTG Sensor Chips — Immobilization of Histidine-Tagged Proteins

The HTG and HTE sensor chips feature a novel tris-NTA (3 x NTA) complex for improved capture of histidine-tagged proteins. This tris-NTA complex has a significantly higher binding stability than the traditional NTA, so minimal ligand leaches off the surface and the sensorgram baseline remains stable. NTA is the traditional method used to capture histidine-tagged proteins but the binding is less tight, causes ligand to leach off the surface, and results in unstable baselines and distorted kinetic results, all of which can lead to an inaccurate fit to a binding model. The tris-NTA complex contains three NTA moieties for improved binding stability and increased binding selectivity to histidine-tagged proteins (Figure 2.7A). The tris-NTA complex is attached to the alginate polymer matrix on the sensor chip and is activated by injecting nickel (II) ions. In order to achieve optimal performance in various applications, the surface density of tris-NTA complex is distinguished in the two different sensor chips, HTG for compact density and HTE for high density (Figure 2.7B and C). The HTG and HTE sensor chips allow easy surface regeneration, chip reuse, and capture of histidine-tagged proteins directly from crude cell lysates. The HTG sensor chip is an ideal choice for protein-protein and protein-peptide interaction analysis, and the HTE sensor chip for protein–small molecule interaction analysis (Figure 2.8). Bio-Rad offers the HTG and HTE reagent kit for use with the HTG and HTE sensor chips.

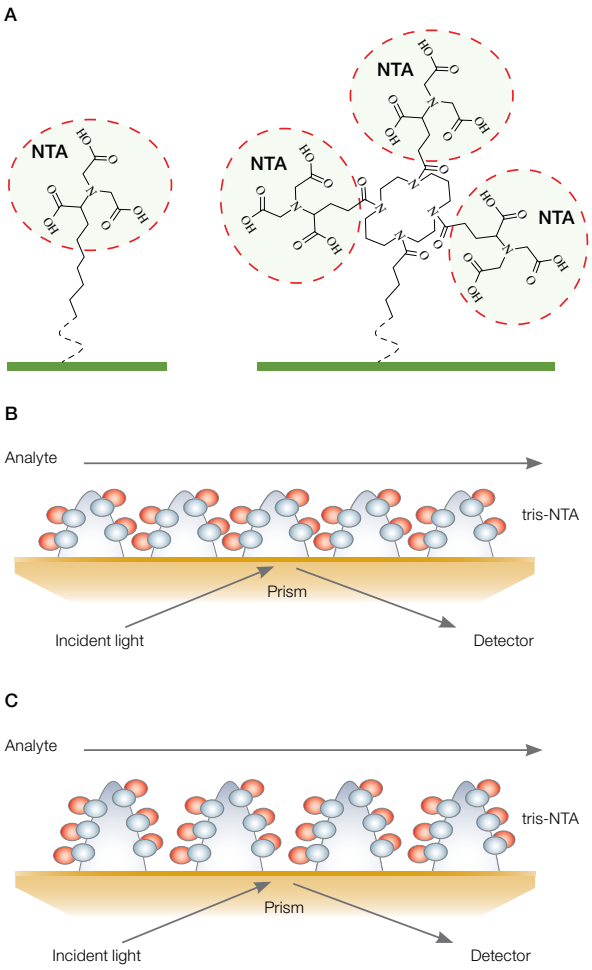


Fig. 2.7. ProteOn HTG and HTE sensor chips. A, structure of surface-bound NTA molecule (left) and tris-NTA molecule (right). Each individual NTA group is circled; **B**, alginate coating modified with compact-density tris-NTA on the HTG sensor chip; **C**, alginate coating modified with high-density tris-NTA on the HTE sensor chip.

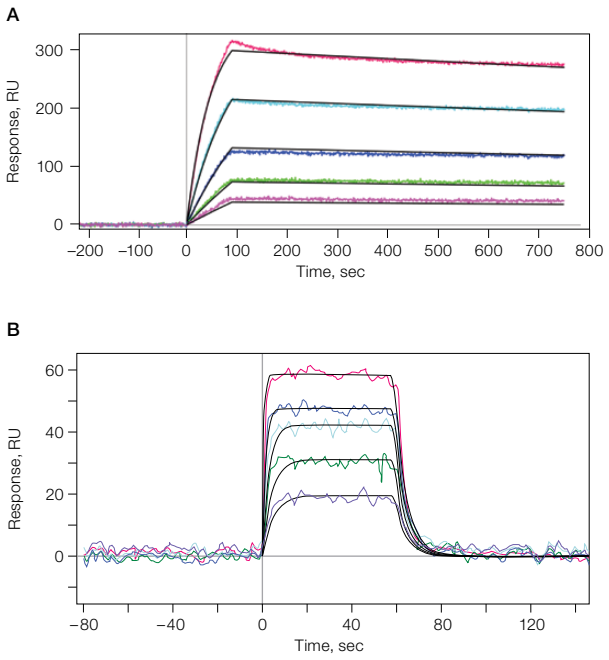


Fig. 2.8. ProteOn HTG and HTE sensor chips. A, sensorgrams of the interaction between the histidine-tagged protein A and human IgG, showing the ability of the HTG sensor chip to resolve high-affinity kinetics requiring long dissociation times. Protein A was captured to approximately 60 RU, and human IgG was injected in a twofold dilution series ranging from 100 nM to 6.3 nM; **B**, sensorgrams of the interaction between histidine-tagged Erk2 (an MAP kinase) and the inhibitor Purvalanol B (432.9 Da), showing that small molecules can be screened using the HTE sensor chip. Erk2 was captured to approximately 12,800 RU, and Purvalanol B was injected in a threefold dilution series ranging from 50–0.62 µM. RU, response units.

2.3.3 ProteOn Sensor Chips for Capturing Lipid Assemblies: Modified GLC and LCP

Biological research focused on biomolecular interactions involving lipid assemblies, such as liposomes and lipoparticles, allows the study of native membrane proteins as well as the role of the lipid bilayer of these assemblies in the activity of the membrane proteins. Analyzing lipid assemblies also provides insights into lipid-protein or lipid–small molecule interactions, answering critical questions in the fields of drug delivery, virology, and signal transduction.

Modified GLC Sensor Chip — Lipophilic Surface Chemistry

The GLC sensor chip can be modified for capturing lipid assemblies. The surface lipophilicity of the chip is adjusted through the amine coupling of an alkyl chain for capturing lipid substances. This capture approach provides the flexibility to control the lipophilicity of the chip surface for customized lipid-based applications.

The modified GLC sensor chip provides a traditional lipophilic surface chemistry for capturing lipid assemblies, which allows for typical lipid-based applications. Bio-Rad offers the GLC lipid kit as an all-in-one kit composed of the GLC sensor chip and the lipid modification kit. The advantages of this application kit are summarized below:

- Lipophilic surface chemistry
- Flexibility in adjusting surface properties
- Good regeneration capability
- Low cost
- High throughput

LCP Sensor Chip — Hydrophilic Surface Chemistry

The LCP sensor chip provides a surface functionalized with NeutrAvidin in a planar configuration that is formed on a self-assembled monolayer (Figure 2.9). It is designed to be used with the ProteOn LCP capturing reagent kit for lipid-protein, lipid–small molecule and membrane protein-protein interaction analysis. The reagent kit activates the chip surface by a biotinylated DNA tag so that the chip is able to capture DNA-labeled lipid assemblies through DNA hybridization. The reagent kit attaches DNA tags to the lipid assemblies in order to anchor them to the chip surface. It is possible to capture two or more layers of lipid assemblies for additional sensitivity. This method of capture allows for lipid-based interaction analysis, including the analysis of membrane proteins embedded in a lipid bilayer (Figure 2.10).

The LCP sensor chip provides a novel hydrophilic surface chemistry for capturing lipid assemblies, which allows for novel lipid-based applications. Bio-Rad offers the liposome capturing kit as an all-in-one kit composed of the LCP sensor chip and the LCP capturing reagent kit. The advantages of this application kit are summarized below:

- Hydrophilic surface chemistry
- Low nonspecific binding
- Multiple layer capture capability
- High regeneration capability
- High throughput

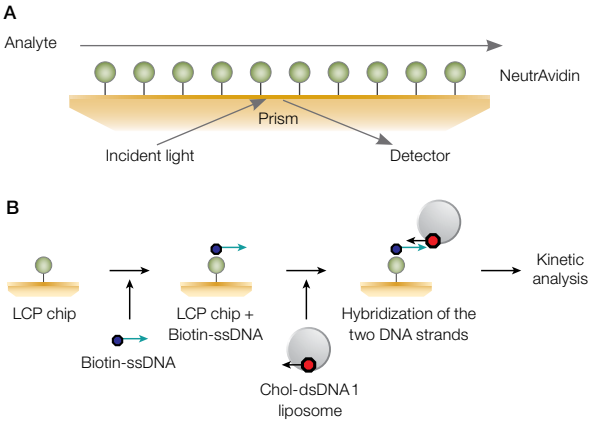


Fig. 2.9. ProteOn LCP sensor chip. A, planar NeutrAvidin-modified self-assembled monolayer on the LCP sensor chip; **B**, workflow for liposome capture using the LCP chip and the LCP capturing reagent kit. Chol-dsDNA 1 and single-stranded biotinylated DNA molecules (biotin-ssDNA) contain complementary DNA sequences. The LCP sensor chip surface is saturated with biotin-ssDNA, and then liposomes incubated with chol-dsDNA 1 are captured to the surface through DNA hybridization. For reagents and techniques used in this workflow, refer to Bio-Rad bulletin 6161.

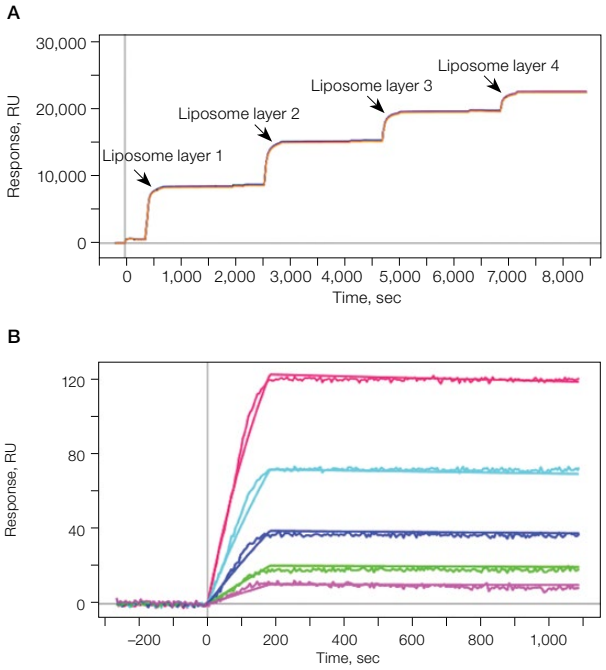


Fig. 2.10. ProteOn LCP sensor chip. A, sensorgram of the stable capture of four 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposome layers. Tagging complementary single stranded DNA molecules to the liposomes facilitates the multiple layer capture capability; **B**, sensorgrams of the interaction between FITC-labeled 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) liposomes captured on the LCP sensor chip and an anti-FITC antibody. FITC-labeled DSPC liposomes were captured to approximately 330 RU, and the anti-FITC antibody was injected in a twofold dilution series ranging from 10–0.63 nM. RU, response units.

2.4 Guidelines for Choosing the Right ProteOn Sensor Chip

Which sensor chip you choose depends on a number of experimental parameters. To help decide you may consider the following questions:

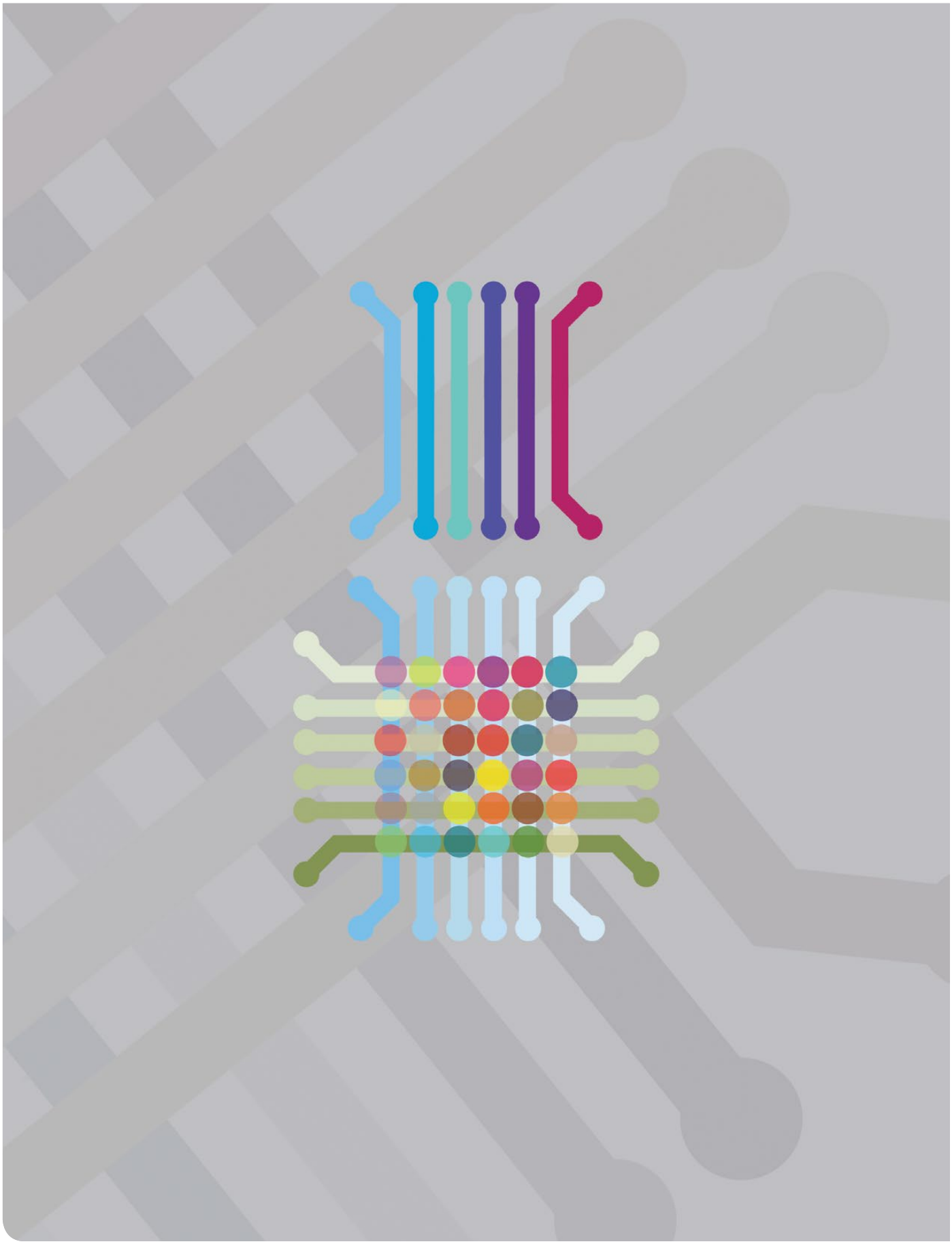
- What is the application?
- What kind of information do I want to get from my ProteOn experiment?
- What type of samples am I working with?
  – Proteins?
  – Peptides?
  – Biotinylated or histidine-tagged proteins?
  – Small molecules?
  – Whole cells?
  – DNA?
- Do I know anything about the affinity (tightness) of binding?
- What are the molecular weights in my sample?

For routine examination of protein-protein interactions such as antibody and antigen, the GLC chip is a good first choice. The GLM or GLH chip can be used as needed if troubleshooting your interaction with the GLC chip suggests that you need a higher capacity surface.

Knowing the molecular weight of your analyte is also crucial for choosing the right sensor chip, as low molecular weight analytes such as small molecules need a higher capacity ligand surface and will benefit from the enhanced sensitivity afforded by the GLH chip.

An additional consideration to take into account when choosing a sensor chip is whether or not your interaction is mass transport limited — in other words, whether or not your interaction has a fast association rate. High capacity surfaces exacerbate a mass transport limited interaction because high density of ligand on the sensor chip depletes the analyte in the surrounding solution very quickly, whereas compact density of ligand mitigates the influence of mass transport by decreasing the rate at which analyte is depleted from the surrounding solution. Thus, the GLC chip with a compact capacity surface may be more suitable than the high capacity GLH chip in dealing with a mass transport limited interaction.

If the ligand is biotinylated or contains a histidine-tag, the NLC, HTG, and HTE chips can be used to immobilize ligand on the chip surface without the need to use amine coupling. An additional benefit of using the NLC, HTG, and HTE chips is that the ligand is immobilized on the chip surface at a specific orientation, as opposed to several random orientations with amine coupling.



CHAPTER 3
Applications

The ProteOn™ XPR36 system monitors many different biomolecular interactions including those between antibodies and antigens, enzymes and substrates, small molecules and drug discovery targets, and whole cells and lipid membranes. The applications are vast and flexible, and the experimental design can be tailored for individual needs. The ProteOn XPR36 system has been widely accepted and used in kinetic screening, protein characterization, protein quantitation, assay optimization, and other novel research fields.

3.1 Overview

Bio-Rad's ProteOn XPR36 protein interaction array system uses surface plasmon resonance (SPR) technology to detect and monitor biomolecular interactions in real time for label-free interaction analysis. Binding events are detected by monitoring the change in the SPR signal, which is proportional to changes in mass at the sensor chip surface over time as an analyte flows through a microfluidic channel and interacts with a target immobilized to the sensor chip. The ProteOn XPR36 system can be used to monitor many different biomolecular interactions including those between antibodies and antigens, enzymes and their substrates/inhibitors, small molecules and drug discovery targets, and whole cells and lipid membranes. SPR technology is flexible; the applications for SPR are vast, and experimental design can be tailored to individual needs. An additional advantage of the ProteOn XPR36 instrument is that unlike some traditional SPR biosensors, the unique XPR™ technology and 6 × 6 interaction array allow for the simultaneous measurement of up to 36 biomolecular interactions (Figure 3.1). XPR technology greatly speeds time to results for traditional kinetic measurements by enabling the patented One-shot Kinetics™ approach, whereby up to six targets are immobilized to the sensor chip surface followed by a single, orthogonal injection of six unique analytes. In addition to traditional kinetic measurements, SPR technology can be used to qualitatively assess biomolecular interaction properties, monitor the quality and/or concentration of biologics, and investigate binding thermodynamics.

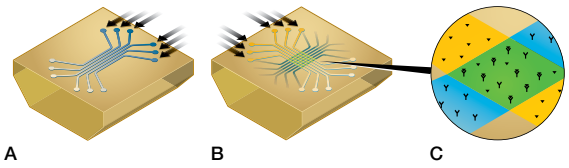


Fig. 3.1. Generation of the 6 x 6 ligand-analyte interaction array. A, six ligands are immobilized in six parallel ligand channels; **B**, six analyte samples are injected into six analyte channels orthogonal to the six ligand channels; **C**, detail of a single ligand-analyte interaction spot (green) showing the positions of the two interspot references (yellow).

The versatility of the microfluidics in the ProteOn XPR36 system makes it an ideal complement to the drug discovery and development workflow for target screening and characterization studies as well as assay design and optimization. The ProteOn XPR36 system is capable of monitoring up to 36 unique biomolecular interactions simultaneously in a variety of configurations depending on the experimental design. The ProteOn XPR36 system can also be used in downstream processes such as protein quantitation for quality control, as SPR can be used to monitor the concentration of small molecules and biologics in crude or impure samples.

3.2 Large and Small Molecule Screening

The versatility of the ProteOn XPR36 platform expands the capabilities of SPR analysis, making this technology ideal for use in several stages of the drug development pipeline. With the rise of antibody therapeutics, there is a need for fast and accurate determination of the affinity of candidate antibodies to their targets. SPR can be used for both quantitative large and small molecule screening and qualitative relative ranking of antibody therapeutics (binding or no binding) during drug development. Small molecules can also be screened for activity and desirable adsorption, distribution, metabolism, and excretion (ADME) properties using the ProteOn XPR36 system. This ability enables the identification of undesirable compounds earlier in the drug discovery process, before considerable time and effort are invested in costly clinical trials.

3.2.1 Antibody Kinetic Screening

The ProteOn XPR36 system is ideal for screening antibody-antigen interactions. The applications in antibody engineering include kinetic screening, epitope mapping, and epitope binding. Epitope mapping is a process by which epitopes on an antigen are pinpointed, and with SPR technology site-directed mutants of an antigen against a given antibody are screened. The ProteOn XPR36 system offers a distinct advantage over other SPR platforms by facilitating One-shot Kinetics: the rapid kinetic screening of antibody targets in a single analyte (antigen) injection. The ProteOn XPR36 system can obtain kinetic constants of single antibody-antigen interaction by simultaneously injecting a full concentration series of an analyte without having to regenerate the ligand surface, in contrast to traditional SPR instruments that allow only sequential injections. In addition, the ProteOn sensor chip can be set up with 36 different target proteins for the acquisition of information-rich kinetic screening data in a high-throughput fashion. This provides a novel method for screening analytes simultaneously against 36 ligands, further increasing the throughput of kinetic screening and enabling a rapid comparison between many interactions. Advantages include:

- Efficient experimental optimization
- Accurate kinetic analysis
- Compatible with crude samples
- High throughput screening

Published Applications

One-Shot Kinetics Approach for Antibody Screening

Bronner V et al. (2005). Rapid and efficient determination of kinetic rate constants using the ProteOn XPR36 protein interaction array system. Bio-Rad Bulletin 3172.

This technical note (Bronner et al. 2005) employs the ProteOn XPR36 system to determine the kinetics of the interaction between interleukin-2 (IL-2) and anti-IL-2 antibody. The experiment was performed using the One-shot Kinetics approach to monitor the interaction between multiple concentrations of the analyte IL-2 and the ligand anti-IL-2 antibody immobilized with multiple conditions in a single analyte injection (Figure 3.2). The 6 x 6 interaction array is used to generate 36 sensorgrams simultaneously. It not only increases throughput but also provides novel referencing options.

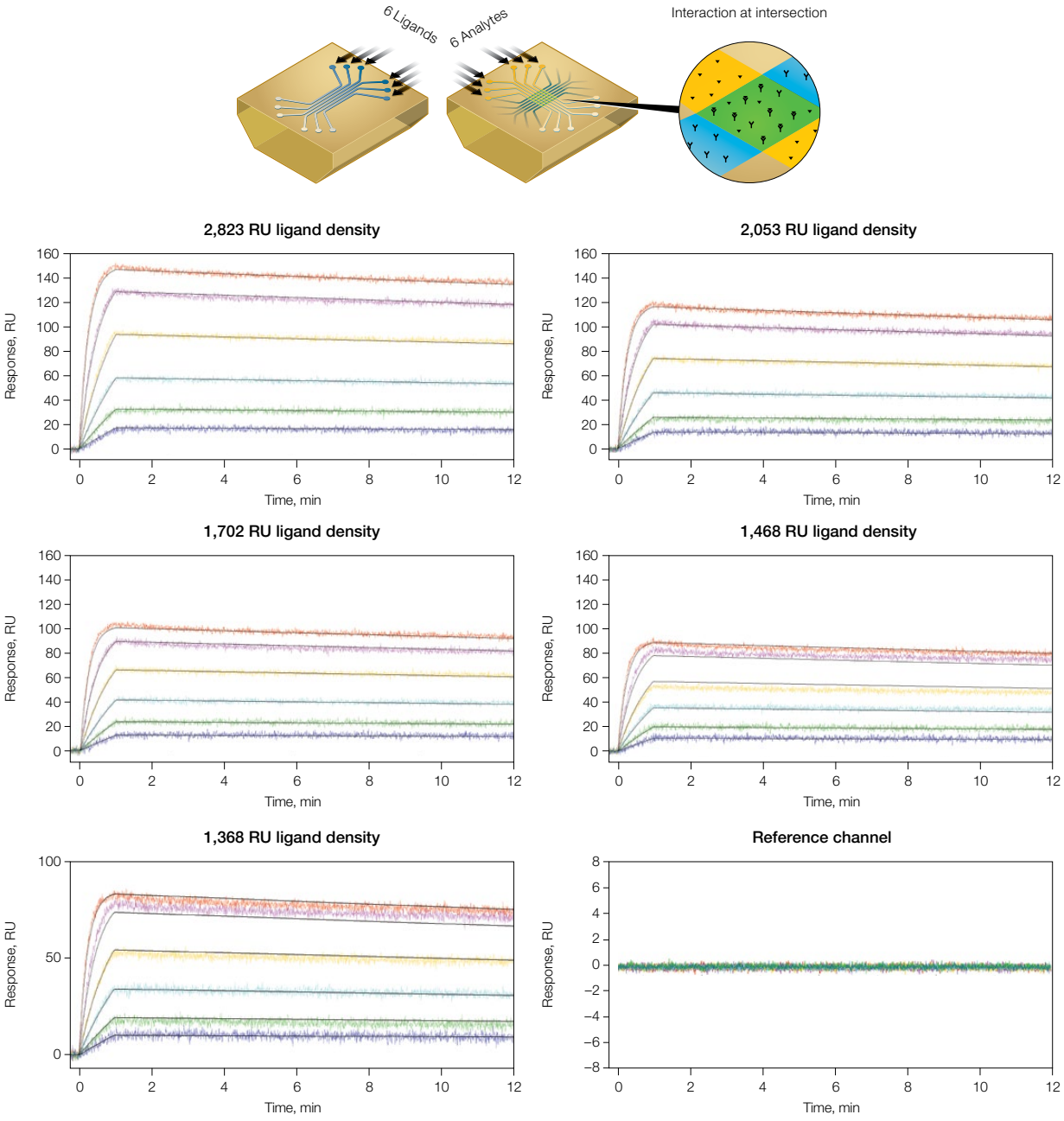


Fig. 3.2. One-shot Kinetics approach for the kinetic analysis of the IL-2 cytokine/IL-2 antibody interaction. Shown are the six sets of six sensorgrams generated in a single analyte injection step. Each set of six sensorgrams displays the responses from the six IL-2 cytokine concentrations [80 nM (—); 40 nM (—); 20 nM (—); 0 nM (—); 5 nM (—); 2.5 nM (—)] interacting with one immobilization level of IL-2 antibody. Sensorgrams are shown for the five levels of IL-2 antibody immobilization (ligand density) and the reference channel. Black lines represent the global fit of the sensorgrams to a 1:1 kinetic interaction model. RU, response units.

Yousef M (2007). Advances in rapid monoclonal antibody screening. Am Biotech Lab 25, 26–28.

The article (Yousef 2007) describes an alternative method for the rapid screening of monoclonal antibodies using multiplexed SPR and the One-shot Kinetics approach of the ProteOn XPR36 system. The ProteOn XPR36 system was used to screen supernatants to identify high-affinity mAb candidates against human IL-12 and hemoglobin E. Over 250 supernatants were screened in 12.5 hr in one experiment, using a single sensor chip. There was no need to purify antibodies from the supernatants prior to analysis.

36-Ligand Array for Antibody Kinetic Screening and Epitope Binding

Abdiche YN et al. (2011). Expanding the ProteOn XPR36 biosensor into a 36-ligand array expedites protein interaction analysis. Anal Biochem 411, 139–151.

Lindquist K (2011). Enhancing throughput of the ProteOn biosensor in antibody screening applications. Bio-Rad ProteOn Webinar 2011 Series.

BioRadiations 133. One Array, 36 Unique Protein Interactions. October 2011.

These articles (Abdiche et al. 2011 and BioRadiations 2011) and the webinar (Lindquist 2011) describe in detail how to create a 36-ligand array for antibody screening (Figure 3.3). This novel use of the ProteOn XPR36 system enables the immobilization of 36 individual ligands to the array surface. In this assay, 36 antibody targets to the same antigen were analyzed using the classical sandwich method and both epitope binding and mapping were performed (Figure 3.4). In the sandwich assay, an antibody is assessed as to whether or not it can bind a preformed antigen-antibody complex and form a sandwich on the surface of a sensor chip. Formation of a sandwich means that the tested antibody recognizes a separate epitope than the immobilized antibody and the absence of a sandwich means that the tested antibody recognizes the same epitope as the immobilized antibody. All these experiments were achieved on a single ProteOn sensor chip in approximately one day.

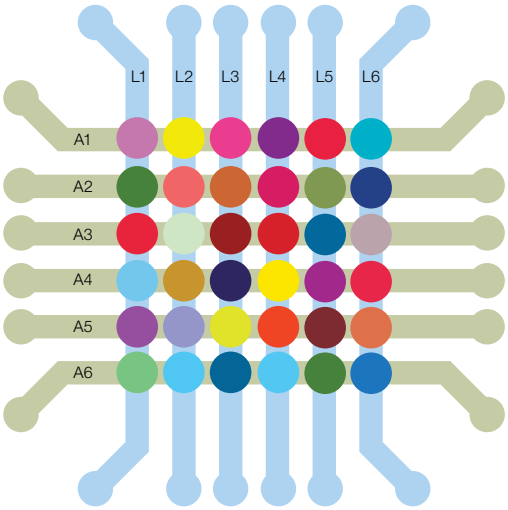


Fig. 3.3. A 36-Ligand array for high throughput screening.

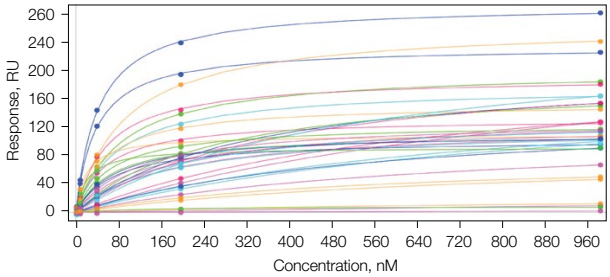


Fig. 3.4. 36 equilibrium binding isotherms generated from dose-response analysis of an analyte over the 36-ligand array. RU, response units.

High Affinity Antigen-Antibody Interaction Analysis

Votsmeier C et al. (2012). Femtomolar Fab binding affinities to a protein target by alternative CDR residue co-optimization strategies without phage or cell surface display. mAbs 4, 341–348.

This article describes the affinity maturation of adalimumab using an approach that employs quantitative screening of soluble Fab fragments with diversification to complementarity-determining region (CDR) and alternative recombination to co-optimize large sets of affinity-improving mutations. The approach achieved ~500 fold affinity improvement and resulted in the first reported femtomolar affinity antibody against protein without display screening. The ProteOn XPR36 system was employed to characterize the binding kinetics and affinity between the antigen and the antibody variants. The result shows the capability of the ProteOn XPR36 system to detect dissociation constants less than 1 x 10^{-6} s^{-1} and equilibrium constants <1 pM, which are the typical limits for label-free biosensors in analyzing high affinity biomolecular interactions.

3.2.2 Epitope Binding and Mapping

Qualitative assessment of binding can be used to rank the relative binding of antibody to targets, map or bind epitopes, and define structure-activity relationships for small molecules. The binding results obtained from SPR analysis of biomolecular interactions can be used to correlate to a particular property of the analyte or the ligand, or to assess cross-reactivity to different biomolecules. Screening antibody targets against antigens with site-directed mutations is used in epitope mapping that leads to improved antibody design. Competitive binding assays such as the classic sandwich assay are used to determine if different antibody molecules recognize the same or different epitopes of an antigen, a process called epitope binding.

- Flexible experimental configuration
- Available for various types of assays
- High-throughput screening

Published Applications

Epitope Binding and Mapping

Abdiche YN et al. (2009). Exploring blocking assays using Octet, ProteOn, and Biacore biosensors. Anal Biochem 386, 172–180.

This article (Abdiche et al. 2009) describes the use of the ProteOn XPR36 system to perform epitope binding using competitive binding assays. Three different assays with different molecular orientations called in-tandem blocking, premix blocking, and classical sandwich assays were performed. The results from the three assays showed strong consistency. When comparing among different label-free biosensors, the ProteOn XPR36 system also showed strong consistency with other platforms utilized.

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

This technical note (Bravman et al. 2007) describes how the ProteOn XPR36 system was applied to the selection, ranking, and epitope mapping of 20 mAb supernatants. The surface, immobilized with anti-mouse mAb, was used to capture supernatant antibody as the ligand, and the analyte IL-9 was injected to analyze the mAb-Ag interactions (Figure 3.5). The throughput of the system was capitalized by capturing five different ligands in parallel and determining the binding kinetic constants in a single injection of five analyte concentrations (Figure 3.6). The four strong binding antibody samples were purified and then epitope mapping was performed. It should be noted that mAb supernatants containing IgM were analyzed. Although the kinetics could not be fitted to the simple Langmuir model, it showed the possibility of qualitative analysis for IgM samples.

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

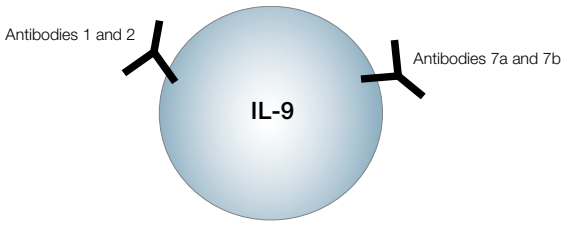


Fig. 3.5. Table of epitope binding.

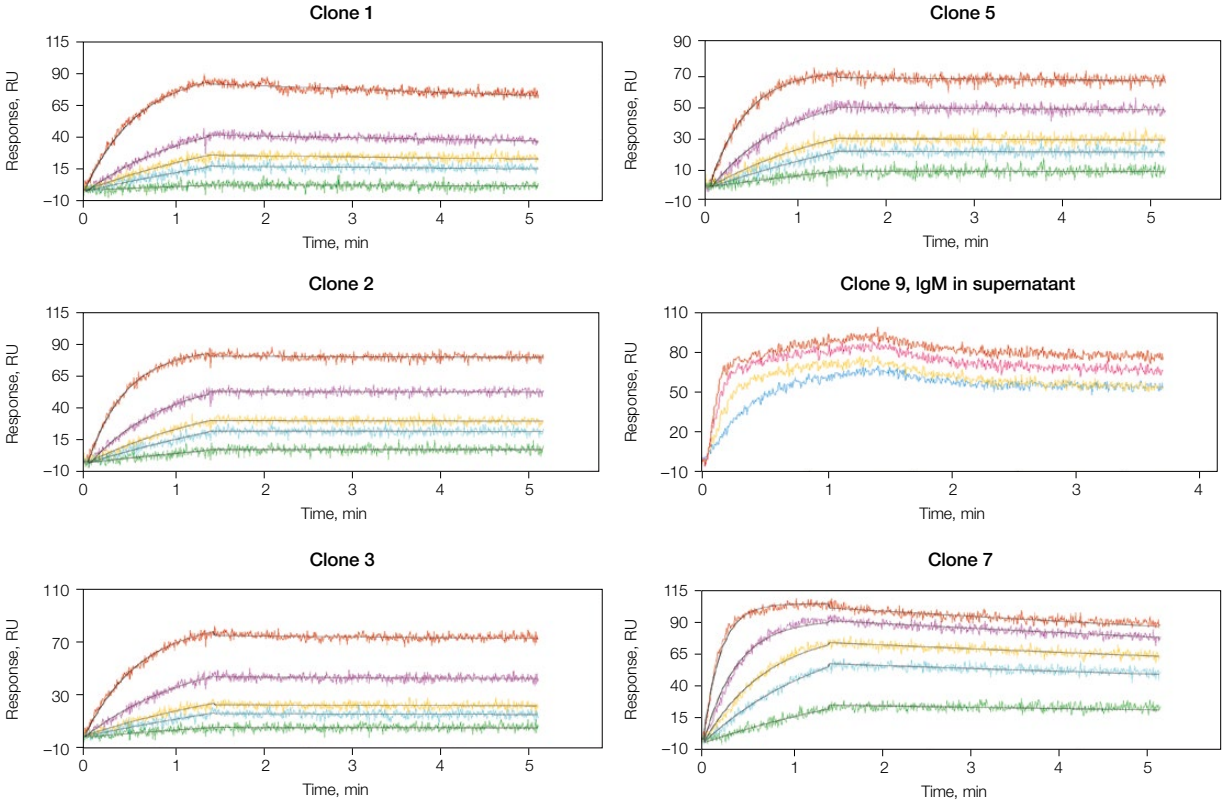


Fig. 3.6. Screening of antibody supernatants. RU, response units.

3.2.3 Quantikinetics

SPR technology is able to determine the concentration of an analyte based on a set of standard samples of known concentrations. Typically a ligand is immobilized on the sensor chip at relatively high density and analyte samples are injected. The initial binding rate of the analyte is measured and correlated directly with analyte concentration. The concentration of an unknown sample is calculated by comparing the binding response under these conditions to a standard curve of binding responses for known concentrations. The parallel fluidics and reproducibility of the ProteOn XPR36 system enable reliable and high-throughput concentration analysis of biological samples for both research applications and manufacturing and quality control processes.

There are two major advantages of SPR over other labeled techniques (such as ELISA) for concentration analysis: (1) label-free SPR eliminates the effort involved in labeling the analyte of interest and (2) using an SPR sensor chip with binding specificity (either by using a capture reagent or immobilizing ligands to the surface) allows for the direct analysis of molecules from crude samples without prior sample purification. The figure illustrates the protein quantitation capability of the ProteOn XPR36 system (Figure 3.7).

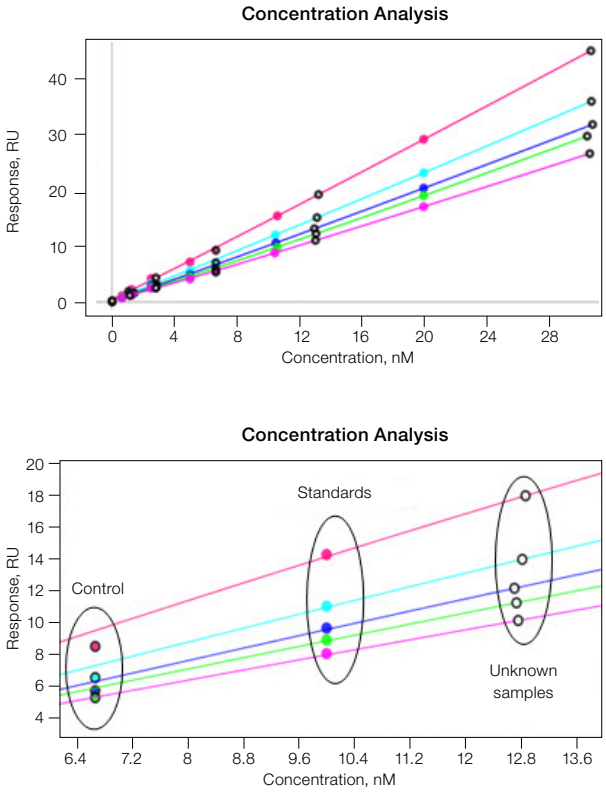


Fig. 3.7. Protein quantitation capability of the ProteOn XPR36 system.

In a typical antibody production workflow, it is necessary to monitor both concentration and kinetics of antibody samples. Utilizing the capabilities of SPR technology, the ProteOn XPR36 system maximizes the power of the unique 6 x 6 experimental configuration to combine concentration and kinetic analysis in a single experiment: this is the so-called quantikinetics workflow. This new workflow will significantly enhance the efficiency and throughput in antibody production.

Published Applications

Analysis of Sample Quantitation and Kinetics in a Single Experiment

Ross G (2012). Antibody quantitation and full kinetic analysis in a single 45 minute experiment using the ProteOn XPR36 system. Bio-Rad ProteOn Webinar Series.

Ross G et al. (2013a). ProteOn XPR36 Quantikinetics: antibody concentration and detailed kinetic analysis in a single experimental cycle. Bio-Rad Bulletin 6411.

Ross G et al. (2013b). ProteOn XPR36 Quantikinetics: antibody concentration and detailed kinetic analysis in a single experimental cycle. Poster presented at Antibody Engineering & Therapeutics, Huntington Beach, USA, Dec. 2013.

This article (Ross et al. 2013a), poster (Ross, 2013b), and webinar (Ross 2012) describe how concentration analysis is performed using an SPR biosensor and how quantikinetics is realized with the ProteOn XPR36 system. Quantikinetics refers to the combination of sample quantitation and kinetic analysis in a single experiment to enhance efficiency and throughput in antibody production (Figure 3.8). For proof of principle, both purified antibody samples and supernatant samples were used to show the workflow of quantikinetics and verify its high performance (Figure 3.9).

$$A_{bulk} \underset{k_t}{\overset{k_t}{\rightleftharpoons}} A_{surface} + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

$$\frac{d[AB]}{dt} \approx k_t[A_{bulk}]$$

Fig. 3.8. SPR technology for concentration analysis.

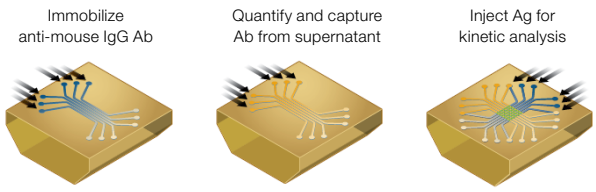


Fig. 3.9. Workflow of quantikinetics.

3.2.4 Drug Compound Screening

An important step in small molecule drug discovery is the screening of large libraries of small molecules for affinity and activity against a protein target. SPR technology is extremely valuable in small molecule drug discovery because it provides information on activity and specificity that allows for the quantitative ranking of lead compounds. The ProteOn XPR36 system has very good sensitivity that allows for the detection of molecules as small as 100 Da. High capacity sensor chips such as the GLH and HTE chips are ready for analyzing small molecules since their high surface capacity for ligand immobilization increases the binding response for a given analyte. In addition, the high throughput and the versatility in experimental design facilitated by the novel 6 x 6 interaction array allows for the production of high quality SPR results using the ProteOn XPR36 ystem.

- High sensitivity (>95 Da)
- High-throughput screening
- Available for fragment screening

Published Applications

Screening of Carbonic Anhydrase Inhibitors

Bravman T et al. (2006). Exploring “One-shot” Kinetics and small molecule analysis using the ProteOn XPR36 array biosensor. Anal Biochem 358, 281–288.

Bravman T et al. (2008). The ProteOn XPR36 array system - high throughput kinetic binding analysis of biomolecular interactions. Cell Mol Bioeng 1, 216–228.

Turner B et al. (2008). Applications of the ProteOn GLH sensor chip: Interactions between proteins and small molecules. Bio-Rad Bulletin 5679.

These articles (Bravman et al. 2006, Bravman et al. 2008, and Turner et al. 2008) describe how the ProteOn XPR36 system was used to investigate the interaction between carbonic anhydrase isozyme (CAII) and a series of known small molecule sulfonamide inhibitors. Kinetic screening results were obtained quickly using the One-shot Kinetics approach (Figure 3.10). The measured affinity of nine inhibitors were consistent with those determined using isothermal titration calorimetry (Table 3.1). Temperature dependence of the kinetics was also measured and Van’t Hoff plot was obtained to determine the thermodynamics of the CAII/carboxybenzenesulfonamide (CBS) interaction. In addition, the binding of a 95 Da methylsulfonamide inhibitor to the ligand CAII was measured, showing the high sensitivity of the ProteOn XPR36 system for small molecule interaction analysis.

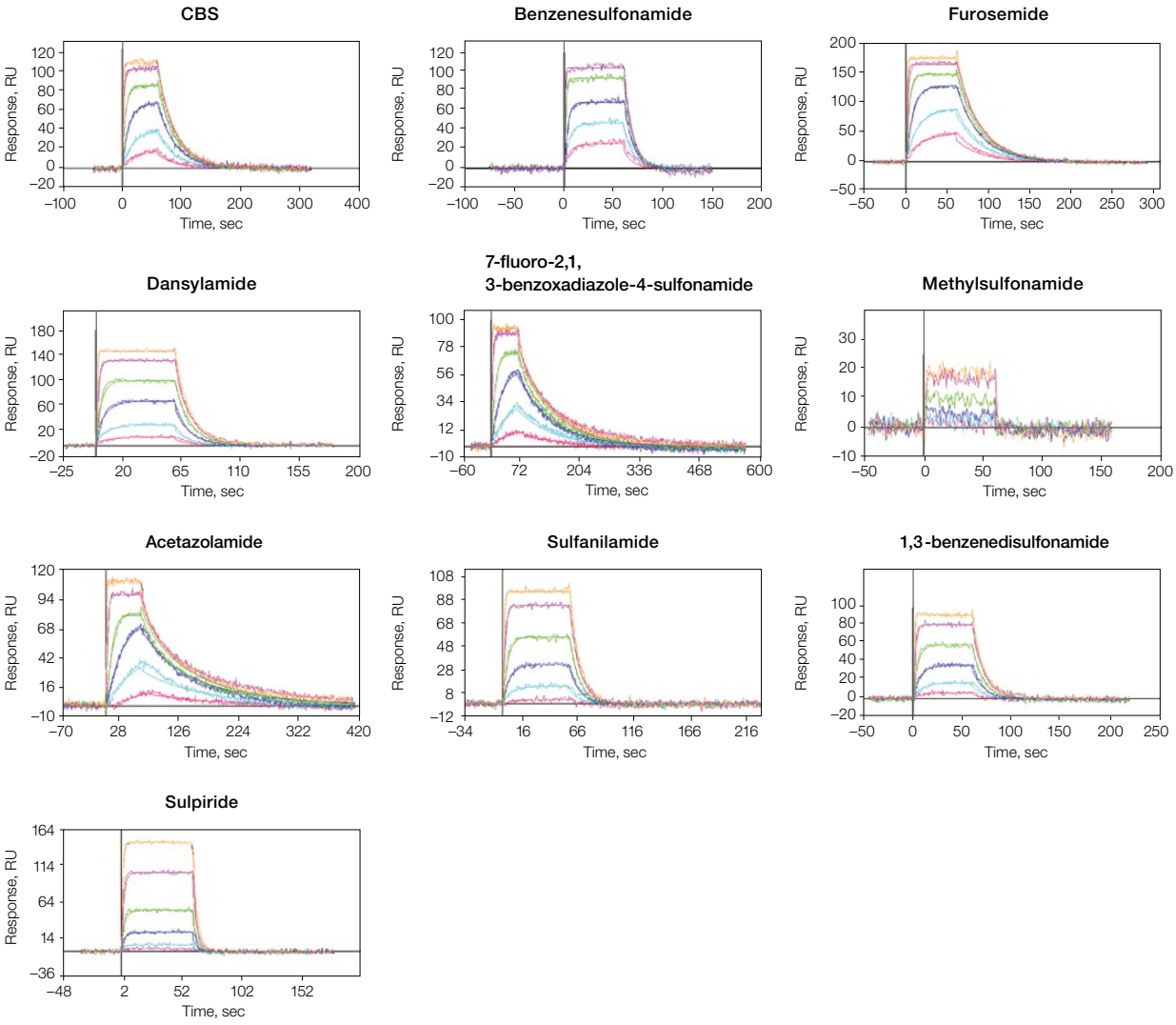


Fig. 3.10. Screening of sulfonamide inhibitors to carbonic anhydrase isozyme. RU, response units.

Table 3.1. Results of the interactions of CAII (MW 29,000) with ten different inhibitors.

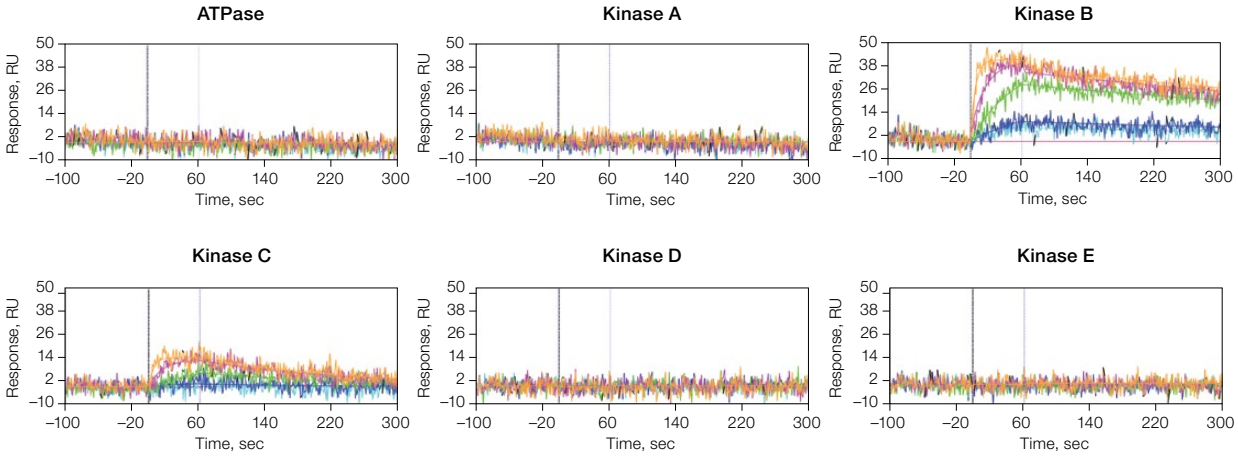
Analyte	MW	Highest Concentration Used, µM	k_a, $M^{-1}sec^{-1}$	k_d, sec^{-1}	K_D, M	R_{max}, RU
Sulpiride	341	250	2.52×10^3	0.26	1.0×10^{-4}	188
Sulfanilamide	172	50	2.40×10^4	0.12	4.8×10^{-6}	112
Furosemide	331	50	5.15×10^4	0.04	7.1×10^{-7}	180
CBS	201	50	2.83×10^4	0.03	1.2×10^{-6}	105
Dansylamide	250	10	1.33×10^5	0.09	6.5×10^{-7}	105
1,3-benzene-disulfonamide	236	10	1.11×10^5	0.09	8.1×10^{-7}	99
Benzenesulfonamide	157	50	1.17×10^5	0.12	1.0×10^{-6}	114
7-fluoro-2,1, 3-benzoxadiazole-4-sulfonamide	217	2	4.64×10^5	0.01	2.8×10^{-8}	82
Acetazolamide	222	2	9.28×10^5	0.02	2.6×10^{-8}	99
Methylsulfonamide	95	2,500	—	—	3.2×10^{-4}	22

Screening of Kinase Inhibitors

Miura T et al. (2010). High-throughput profiling of kinase inhibitors selectivity using the ProteOn XPR36 protein interaction array system. Bio-Rad Bulletin 5960.

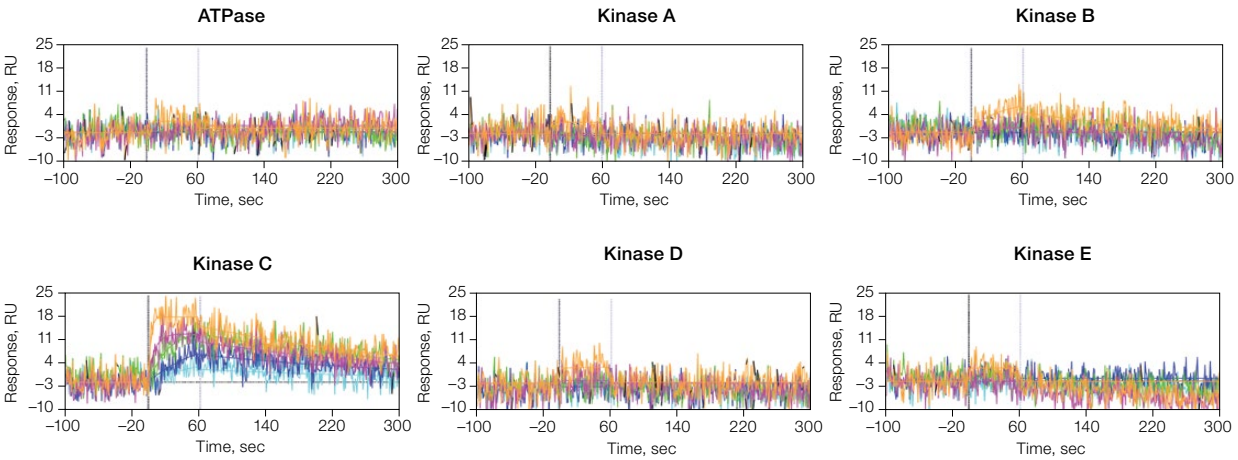
This technical note (Miura et al. 2010) describes the use of the One-shot Kinetics approach of the ProteOn XPR36 system to generate K_D values during the screening of small molecule kinase inhibitors against five known important kinase targets. The biotinylated kinases and one ATPase were immobilized and a concentration series of the inhibitors were injected (Figure 3.11). The assay was evaluated using Z'-factor analysis and it was shown that an R_{max} of 17 RU was sufficient to achieve consistent results.

Inhibitor A



	K_D, nM	k_a, $M^{-1}s^{-1}$	k_d, s^{-1}
Kinase B	8	1.75×10^5	1.4×10^{-3}
Kinase C	34	1.60×10^5	5.5×10^{-3}

Inhibitor B



	K_D, nM	k_a, $M^{-1}s^{-1}$	k_d, s^{-1}
Kinase C	8.4	3.9×10^5	3.2×10^{-3}

Fig. 3.11. Screening of two inhibitors' binding kinetics to the six different ligands. Inhibitors A and B concentrations are 1,000 (—), 333 (—), 111 (—), 37 (—), and 12 µM (—). RU, response units.

Tabul M et al. (2010). Rapid, high-throughput screening of protein kinase inhibitors using the ProteOn XPR36 protein interaction array system. Bio-Rad Bulletin 5965.

This technical note (Tabul et al. 2010) describes the rapid optimization of immobilization conditions for protein kinase targets (p38 and Erk2) to ensure that the immobilized molecules remain in an active state.

Compounds from two small molecule kinase inhibitor libraries were screened against each kinase target to identify binding hits (Figures 3.12 and 3.13). Detailed kinetic analysis of the positive binding compounds was conducted to determine the binding constants. Screening of 110 compounds took only 5 hr, and all the work was completed on a single sensor chip.

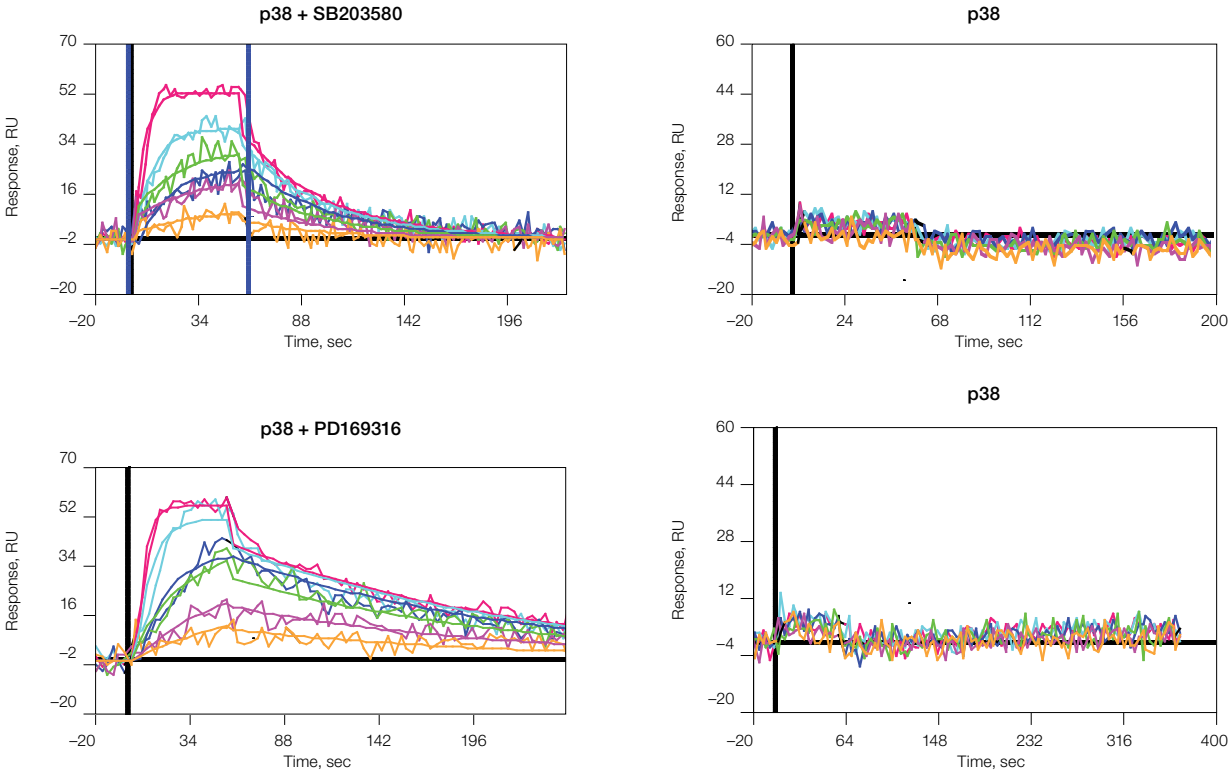


Fig 3.12. Ligand-protected immobilization (left), with the addition of inhibitors SB203580 and PD169316 shows preserved p38 kinase activity compared to direct immobilization (right). RU, response units.

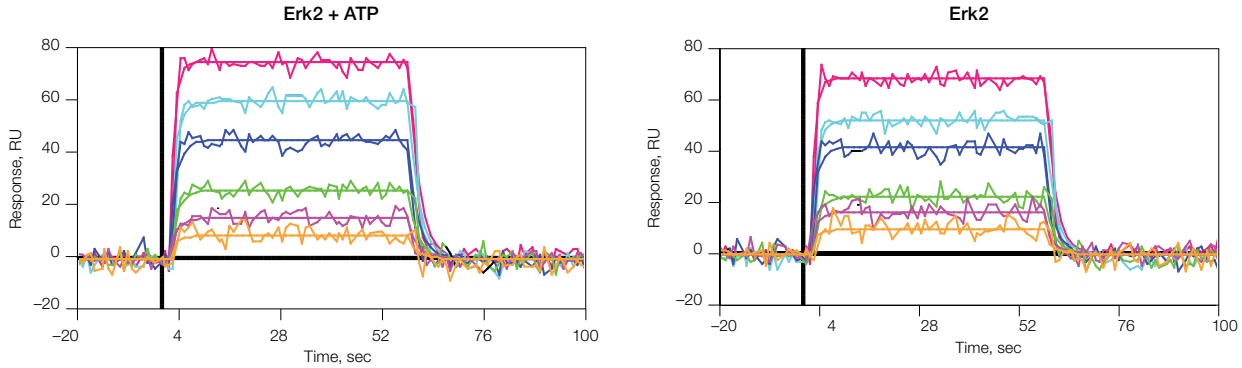
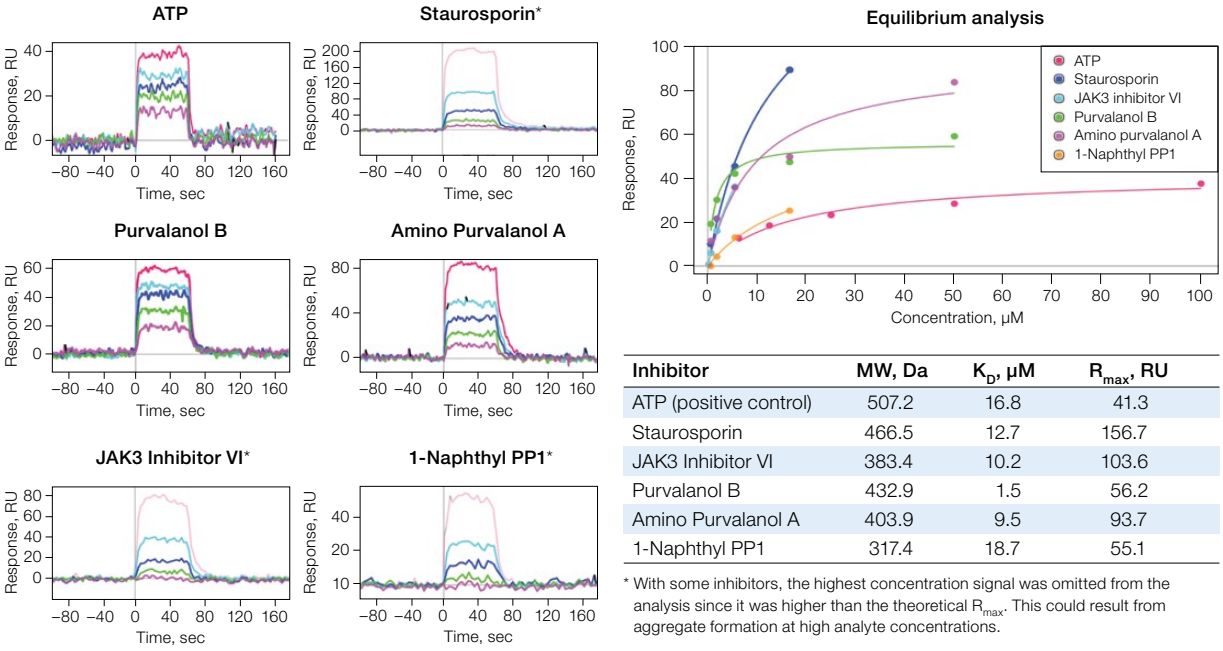


Fig. 3.13. Ligand-protected immobilization (left), with the addition of ATP does not impact Erk2 kinase activity compared to direct immobilization (right). RU, response units.

Luo R et al. (2011). Novel ProteOn sensor chips with high stability and high selectivity for label-free polyhistidine-tagged protein interaction analysis. Poster presented at Antibody Engineering and Therapeutics, San Diego, Dec. 2011.

The histidine tag is one of the most widely used tags in protein purification. Recombinant proteins containing the histidine tag are easily captured by a tris-nitrilotriacetate (tris-NTA) surface. Bio-Rad offers the HTG and HTE chips for capturing histidine-tagged proteins for interaction analysis. The chips employ a novel tris-NTA surface chemistry, which provides higher stability and selectivity in capturing histidine-tagged proteins, compared to the traditional NTA surface chemistry, and allows for surface regeneration. This surface intercalates at three points with a histidine-tagged protein rather than a single point for the NTA surface. The HTG chip is ideal for the analysis of protein-protein and protein-peptide interactions, and HTE chip for protein–small molecule interactions. In this work (Luo et al. 2011), small molecules binding to histidine-tagged Erk2 were screened based on the equilibrium analysis (Figure 3.14).



Inhibitor	MW, Da	K_D, µM	R_{max}, RU
ATP (positive control)	507.2	16.8	41.3
Staurosporin	466.5	12.7	156.7
JAK3 Inhibitor VI	383.4	10.2	103.6
Purvalanol B	432.9	1.5	56.2
Amino Purvalanol A	403.9	9.5	93.7
1-Naphthyl PP1	317.4	18.7	55.1

* With some inhibitors, the highest concentration signal was omitted from the analysis since it was higher than the theoretical R_{max}. This could result from aggregate formation at high analyte concentrations.

Fig. 3.14. Experiment screening small molecule inhibitors against a polyhistidine-tagged enzyme. The high-quality results show the potential of using the HTE chip in drug screening. The ligand was captured on the HTE chip surface and the response reached ~12,700 RU. The capture uniformity and the baseline stability were excellent. Fixation of the ligand to the surface is not necessary. Analyte concentrations, ATP: 100, 50, 25, 12.5, and 6.2 µM; Staurosporin: 50, 16.7, 5.6, 1.9, and 0.6 µM; JAK3 Inhibitor VI: 16.7, 5.6, 1.9, 0.6, and 0.2 µM; Purvalanol B: 50, 16.7, 5.6, 1.9, and 0.6 µM; Amino purvalanol A: 50, 16.7, 5.6, 1.9, and 0.6 µM; 1-Naphthyl PP1: 50, 16.7, 5.6, 1.9, and 0.6 µM. RU, response units.

Fragment Screening

Dolezal O (2013). Lead discovery: Screening and characterization using multiplexed SPR. Bio-Rad ProteOn Webinar Series.

Peat TS et al. (2012). Small molecule inhibitors of the LEDGF site of Human Immunodeficiency Virus Type 1 Integrase identified by fragment screening and structure based design. PLoS ONE 7: e40147.

Popplewell J (2013). SPR-based fragment screening and small molecule affinity analysis using ProteOn XPR36 system. BioRadiations Sep. 2013.

Fragment-based lead discovery, or fragment screening, is a method used for finding lead compounds in drug discovery. It is based on identifying small chemical fragments, which may bind weakly to the biological target, and deriving or combining them to build a lead compound with higher binding affinity. An SPR biosensor is one of the major tools used in fragment screening. These articles and the webinar report different fragment screening experiments performed with the ProteOn XPR36 system (Figure 3.15).

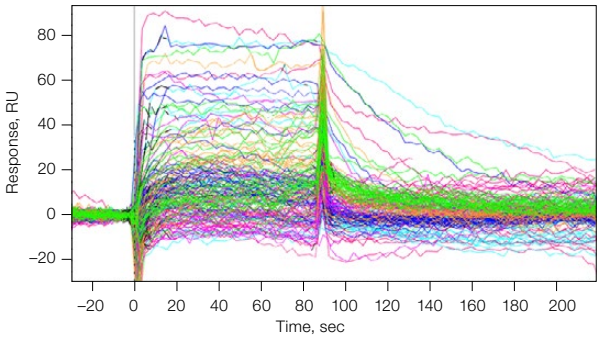


Fig. 3.15. Screening of 260–360 Dalton small molecules for fragment-based lead discovery. RU, response units.

3.3 Biomolecule Characterization

Unlike endpoint assays such as ELISA that measure a binding response after reaching equilibrium, SPR allows for the detection of binding events in real time and the accurate measurement of both association (k_a) and dissociation (k_d) rate constants. This additional information can be helpful in designing potent agonists/antagonists with a fast k_a and slow k_d or for understanding quantitative structure-activity relationships of small molecules. The unique One-shot Kinetics approach of the ProteOn XPR36 system allows for robust kinetic characterization from a single analyte injection. In addition, biomolecular interaction thermodynamics can be characterized by using the ProteOn XPR36 system to quantify kinetics at different temperatures.

3.3.1 Structural Biology

The ProteOn XPR36 system has wide applications in structural biology and biophysics to pinpoint structures in small molecules and regions on proteins that are responsible for binding. Because SPR obtains information on both the association and dissociation phases of the biomolecular interaction, it allows for discovery of quantitative structure-activity relationships based on the effects of specific structures. SPR benefits include:

- Highly efficient mutagenesis workflow for structural biology
- Compatible with crude samples
- Flexible experimental configuration
- Efficient experimental optimization

Published Applications

Characterization of the Interaction of TEM1 and BLIP

Bronner V et al. (2005). Mechanisms of protein-protein binding: double-mutant cycle analysis using the ProteOn XPR36 system. Bio-Rad Bulletin 5358.

Reichmann D et al. (2005). The modular architecture of protein-protein binding interfaces. Proc Natl Acad Sci USA 102, 57–62.

This technical note (Bronner et al. 2005) and article (Reichmann et al. 2005) employ the ProteOn XPR36 system to uncover important residues for the interaction of two proteins, TEM1 beta-lactamase (TEM1) and its inhibitor beta-lactamase inhibitor protein (BLIP). In this study, double-mutant cycle (DMC) analysis was used along with the innovative One-shot Kinetics approach in the ProteOn XPR36 system. The DMC analysis is an excellent tool to investigate the structure, mechanism, and dynamics of protein-protein interactions. Multiple mutants of TEM1 and BLIP were analyzed against each other to determine the contribution of residues toward the stability of the TEM1/BLIP interaction.

Bronner V et al. (2005). Analysis of multiple protein-protein interactions using the ProteOn XPR36 protein interaction array system. Bio-Rad Bulletin 5368.

This technical note (Bronner et al. 2005) employs the ProteOn XPR36 system to uncover important residues for the interaction of two proteins, TEM1 beta-lactamase (TEM1) and its inhibitor beta-lactamase inhibitor protein (BLIP) (Figure 3.16). In this study, the interaction analysis of TEM1 mutants and BLIP was used to construct a picture of the TEM1/BLIP binding interface. The innovative One-shot Kinetics approach in the ProteOn XPR36 system was employed to achieve kinetic analysis with high accuracy and efficiency (Table 3.2). The kinetic analysis could be used to derive the binding energetics and obtain the architecture of the binding interface.

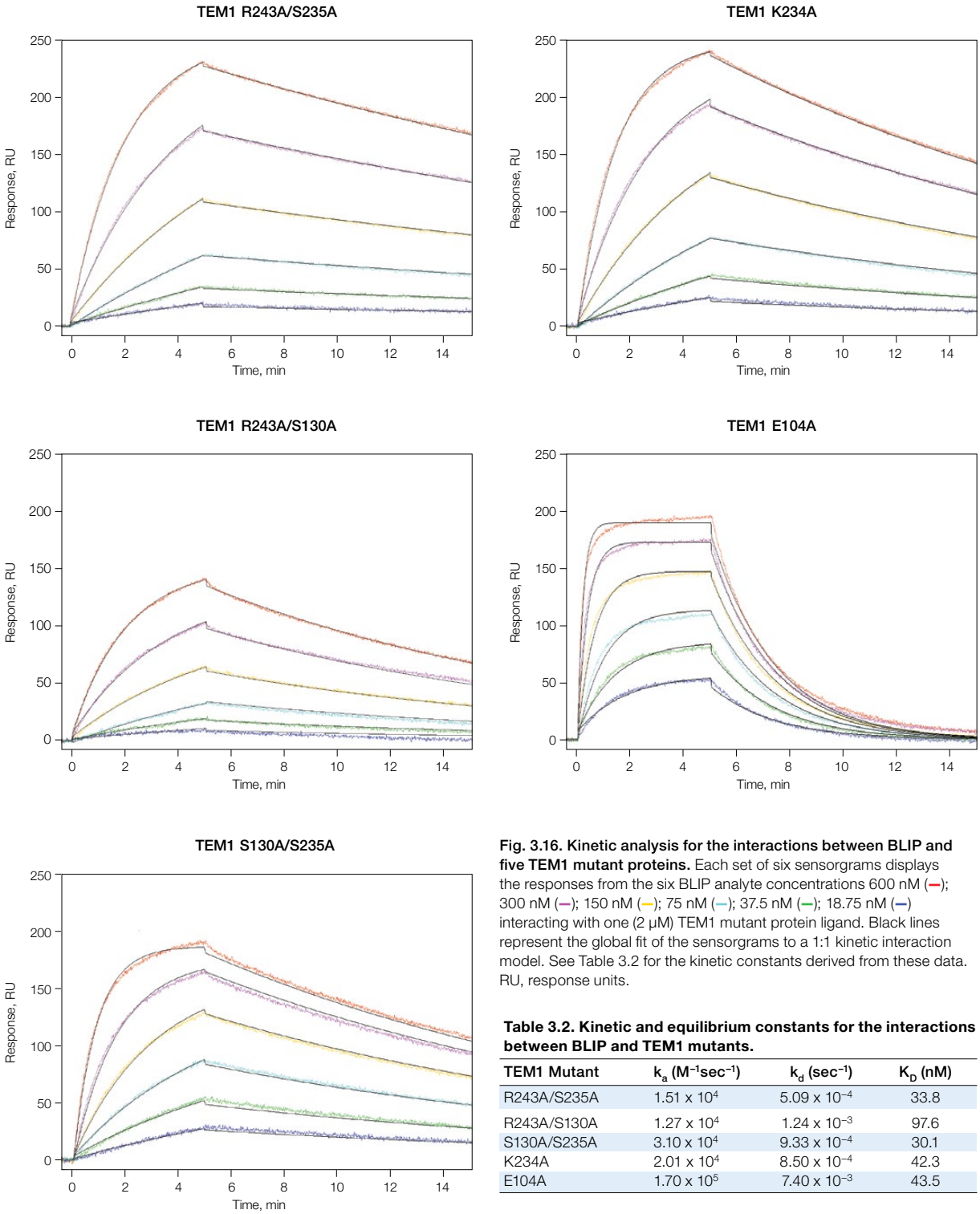


Fig. 3.16. Kinetic analysis for the interactions between BLIP and five TEM1 mutant proteins. Each set of six sensorgrams displays the responses from the six BLIP analyte concentrations 600 nM (—); 300 nM (—); 150 nM (—); 75 nM (—); 37.5 nM (—); 18.75 nM (—) interacting with one (2 µM) TEM1 mutant protein ligand. Black lines represent the global fit of the sensorgrams to a 1:1 kinetic interaction model. See Table 3.2 for the kinetic constants derived from these data. RU, response units.

Table 3.2. Kinetic and equilibrium constants for the interactions between BLIP and TEM1 mutants.

TEM1 Mutant	k_a ($M^{-1}sec^{-1}$)	k_d (sec^{-1})	K_D (nM)
R243A/S235A	1.51×10^4	5.09×10^{-4}	33.8
R243A/S130A	1.27×10^4	1.24×10^{-3}	97.6
S130A/S235A	3.10×10^4	9.33×10^{-4}	30.1
K234A	2.01×10^4	8.50×10^{-4}	42.3
E104A	1.70×10^5	7.40×10^{-3}	43.5

Characterization of the β-Amyloid Peptide Assemblies in Alzeheimer's Disease Research

Di Fede G et al. (2009). A recessive mutation in the APP gene with dominant-negative effect on amyloidogenesis. Science 323, 1473–1477.

This article (Di Fede et al. 2009) reports the characterization of an amyloid precursor protein mutation (A673V) that causes disease only in the homozygous state whereas heterozygous carriers are unaffected. The ProteOn XPR36 system was used to determine the binding of wild-type and mutated Aβ(1-40) or Aβ(1-6) to $Aβ(1-40)_{wt}$ fibrils. The results showed no difference between $Aβ(1-40)_{wt}$ and $Aβ(1-40)_{mut}$ binding to $Aβ(1-40)_{wt}$ fibrils. However, the amino-terminal fragment $Aβ(1-6)_{mut}$ showed greater ability to bind to $Aβ(1-40)_{wt}$ fibrils than did $Aβ(1-6)_{wt}$, indicating that the A-to-V substitution in the amino acid sequence facilitates the interaction between mutant and wild-type Aβ peptides. The observation is consistent with the previously reported molecular mechanism of Aβ aggregation.

Di Fede G et al. (2012). Good gene, bad gene: New APP variant may be both. Prog Neurobiol 99, 281–292.

Gobbi M (2012). Novel application of SPR to study amyloidogenic peptides and proteins. Bio-Rad ProteOn Webinar Series.

Stravalaci M et al. (2010). Use of surface plasmon resonance to study the elongation kinetics and the binding properties of the highly amyloidogenic Aβ(1-42) peptide, synthesized by depsi-peptide technique. Biosens Bioelectron 26, 2772–2775.

Taylor M et al. (2010). Development of a proteolytically stable retro-inverso peptide inhibitor of beta-amyloid oligomerization as a potential novel treatment for Alzheimer's disease. Biochemistry 49, 3261–3272.

These articles (Di Fede et al. 2012, Stravalaci et al. 2010, and Taylor et al. 2010) and the webinar (Gobbi 2012) outline an SPR-based approach to investigate the elongation of Aβ fibrils and an immunoassay to analyze the formation of Aβ oligomers.

The interaction between Aβ monomer, Aβ oligomers, and Aβ fibrils could be characterized with kinetic analysis using the ProteOn XPR36 system. When Aβ fibrils were immobilized on the surface of sensor chips, Aβ monomers and the mixture with inhibitors could be applied to investigate the elongation of Aβ fibrils and evaluate the inhibitors.

Using the ProteOn XPR36 system, aliquots of a solution of Aβ(1-42) monomer were taken at different time points, and injected to the system to analyze the interaction with 4G8 antibody. As time passed, the aliquot contained varying concentrations of synthetic Aβ(1-42) monomer, and spontaneously formed Aβ(1-42) oligomers and Aβ(1-42) fibrils. The interaction sensorgrams were deconvoluted to obtain the concentration ratio between Aβ(1-42) monomer and Aβ(1-42) oligomers that was verified by combining SPR with chromatography (Figure 3.17). It was shown that the immunoassay could be used to analyze the formation of Aβ oligomers, thus allowing the evaluation of inhibitors and effects of Aβ mutations, as well as the potential detection of native Aβ oligomers in biological samples.

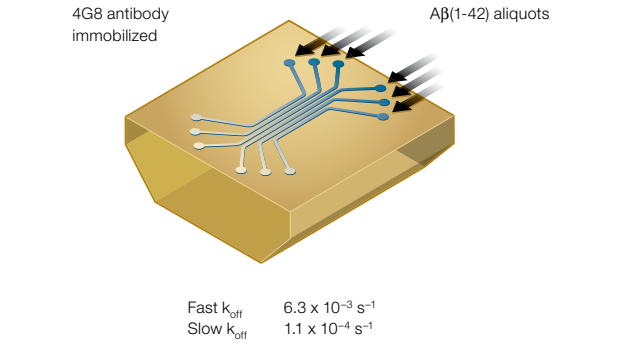


Fig. 3.17. Binding of Aβ(1-42) aliquots taken from the same solution at different time points (0–72 hr) resolving the fast k_{off} Aβ(1-42) monomers and slow k_{off} Aβ(1-42) oligomers. Experimental data: binding species 1 + binding species 2 (—); binding species 1 (fast k_{off}) (····); binding species 2 (slow k_{off}) (– –). RU, response units.

Balducci C et al. (2010). Synthetic amyloid-beta oligomers impair long-term memory independently of cellular prion protein. Proc Natl Acad Sci USA 107, 2295–2300.

Biasini E (2013). Using SPR to characterize the interaction between the cellular prion protein and Aβ oligomers. Bio-Rad ProteOn Webinar Series.

Fluharty BR et al. (2013). An N-terminal fragment of the prion protein binds to amyloid-β oligomers and inhibits their neurotoxicity in vivo. J Biol Chem 288, 7857–7866.

These articles (Balducci et al. 2010 and Fluharty et al. 2013) and the webinar (Biasini 2013) describe research on a pathway related to Alzeheimer's disease using the ProteOn XPR36 system. The interaction between Aβ assemblies and cellular prion protein (PrP^C) was investigated. In order to characterize the binding site on PrP^C, the PrP^C fragments PrP^{23-230}, N1 terminus, and C1 terminus were captured on the chip surface using an anti-Myc antibody. Aβ monomer and Aβ oligomers were injected in the parallel flow channels for interaction analysis (Figure 3.18). Further characterization showed the interaction between the N1 terminus and transient Aβ assemblies. It was discovered that the N1 terminus of PrP^C was necessary and sufficient to facilitate the binding to Aβ oligomers and could block some detrimental effects, thus it represents a new generation of therapeutic agents for Alzeheimer's disease.

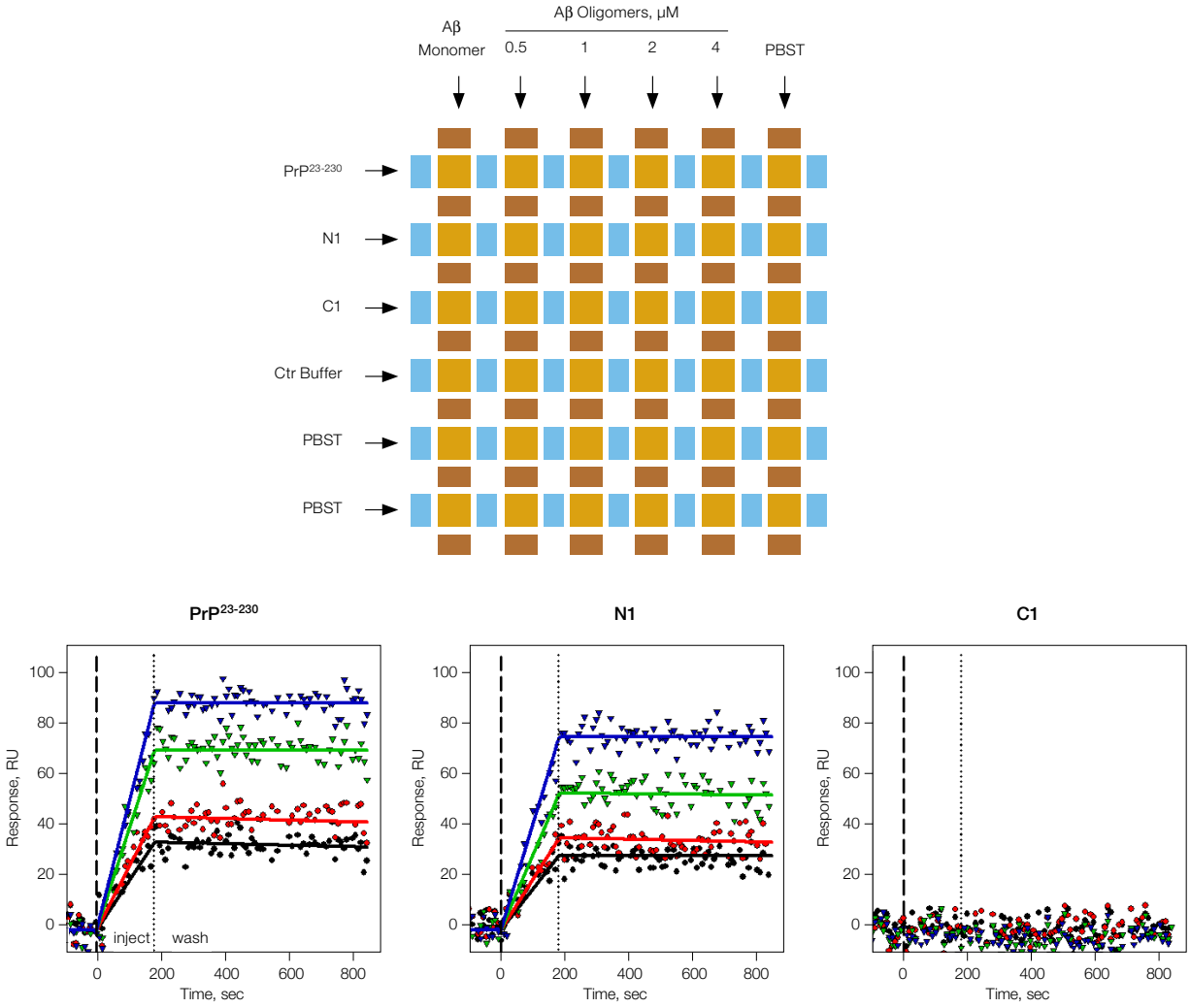


Fig. 3.18. Interaction analysis between PrP^c fragments PrP^{23-230}, N1 terminus, C1 terminus, and Aβ assemblies. 4 μM Aβ oligomers (▲); 2 μM Aβ oligomers (▼); 1 μM Aβ oligomers (●); 0.5 μM Aβ oligomers (●). RU, response units.

Characterization of the Interaction Between Proteins and Phosphoinositides

Sommer L (2012). Identification and characterization of novel phosphoinositide binding proteins using SPR. Bio-Rad ProteOn Webinar Series.

Sommer L (2012). Using DNA hybridisation to capture liposomes for SPR based detection of phosphoinositide-protein interactions. Poster presented at PEGS, Boston, USA, Apr. 2012.

Phosphoinositides are important lipid signaling molecules found in all cellular membranes and also in a non-membranous (endo-nuclear) form within the nucleus. Phosphoinositide signaling is mediated through interaction with proteins that contain specific phosphoinositide binding domains. The ProteOn XPR36 system was used to analyze some phosphoinositide-protein interactions of interest (Figure 3.19).

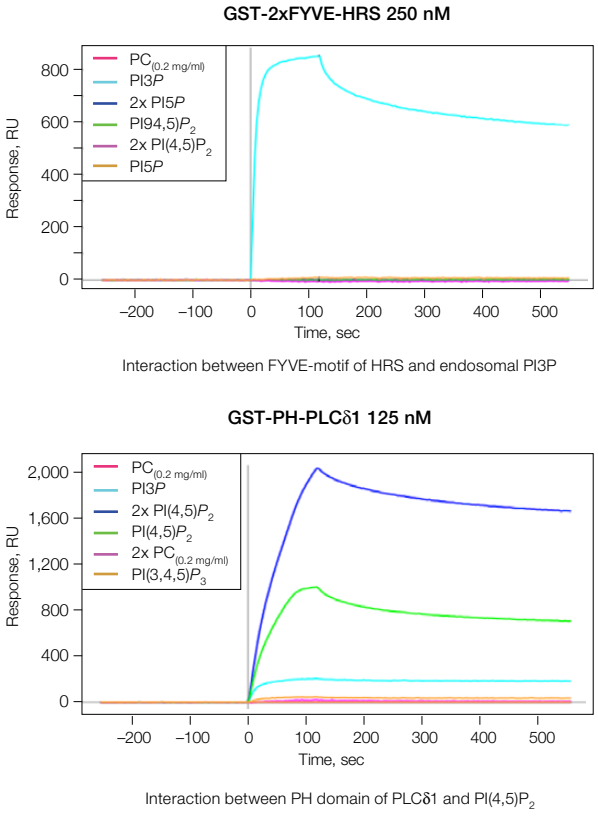


Fig. 3.19. Interaction of phosphoinositides with various proteins containing phosphoinositide binding domains and proteins that lack a phosphoinositide binding domain. RU, response units.

Lekomtsev S et al. (2012). Centralspindlin links the mitotic spindle to the plasma membrane during cytokinesis. Nature 492, 276–279.

The article (Lekomtsev et al. 2012) describes the research on the molecular mechanism of cytokinesis. A set of tools were used to study the connection between mitotic spindle and plasma membrane during cytokinesis. It was discovered that the C1 domain of the centralspindlin subunit MgcRacGAP associated with the plasma membrane by interacting with polyanionic phosphoinositide lipids. The ProteOn XPR36 system was used in the analysis of the binding of MgcRacGAP C1 domain to phosphatidylethanolamine vesicles containing 5% PtdIns(4)P or PtdIns(4,5)P_2. The model of MgcRacGAP C1 domain-membrane interaction was established (Figure 3.20).

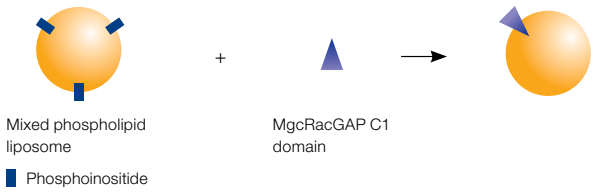


Fig. 3.20. Analysis of the binding of MgcRacGAP C1 domain to phosphatidylethanolamine vesicles containing 5% phosphatidylinositol-4-phosphate (PtdIns(4)P) or phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2).

Characterization of the Interaction of IL-1 R1 and IL-1β in the Presence of RAcP

Issafras H (2011). Antibody characterization using the ProteOn XPR36 system, a multiplexed SPR biosensor. Bio-Rad ProteOn Webinar Series.

This webinar reports the work that investigated the binding of interleukin-1β (IL-1β) and the receptor (IL-1 R1) in the presence of the receptor accessory protein (RAcP) (Issafras 2011). Utilizing the parallel channel experimental configuration in the ProteOn XPR36 system, interaction surfaces with different mixing ratios of the two proteins and reference surfaces were established and simultaneously analyzed with IL-1β was implemented. The synergy of the two proteins was observed in the sensorgrams. The synergy resulted in the fitting to the 1:1 Langmuir model while the single receptor showed more complicated binding mechanisms (Figure 3.21).

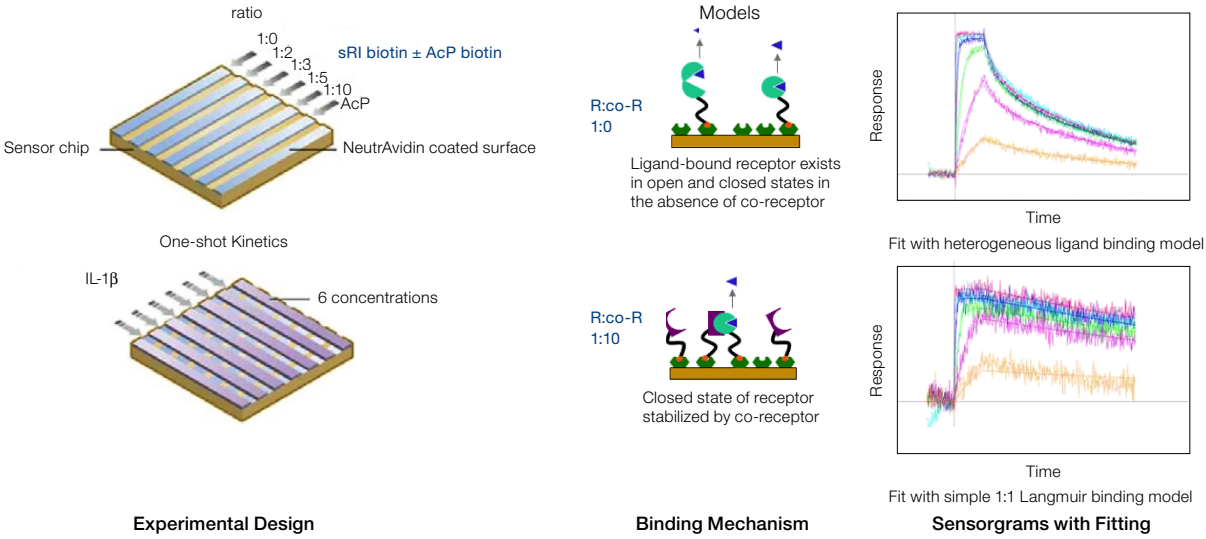


Fig. 3.21. The experimental design, binding mechanism, and sensorgrams with fitting in the analysis between IL-1β, IL-1 R1, and RAcP.

Characterization of the Interaction of Ubiquitin and Ubiquitin Binding Domains

Marchese A (2011). Identification of a novel ubiquitin binding domain by SPR. Bio-Rad ProteOn Webinar Series.

Ubiquitin modification of proteins is involved in proteasomal degradation and also serves many non-proteasomal functions in a wide variety of biological functions. The functional outcome of ubiquitin modification is dependent upon noncovalent interactions with ubiquitin binding domains. This webinar outlines SPR experiments, in which a biotinylated peptide, encompassing a novel ubiquitin binding domain, was immobilized on an NLC chip, and single and polymerized forms of ubiquitins were used as analytes (Marchese 2011). Dose-response sensorgrams of the interaction of the peptide and the analytes were observed (Figure 3.22).

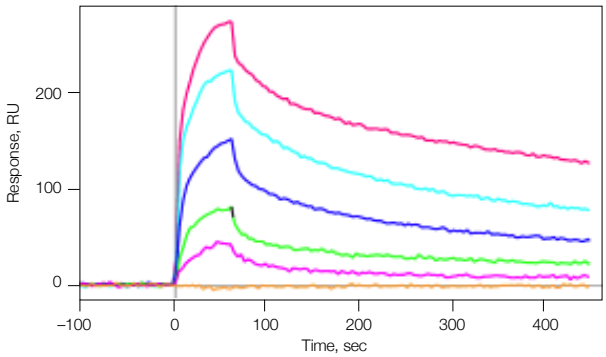


Fig. 3.22. Sensorgrams showing dose-response analysis of the interaction between a peptide and ubiquitins. RU, response units.

High Affinity Proteasome-Activator Interaction Analysis

Stadtmueller BM et al. (2010). Structural models for interactions between the 20S proteasome and its PAN/19S activators. J Biol Chem 285, 13–17.

This article (Stadtmueller et al. 2010) describes the structural models of the binding between the PAN/19S activators and the 20S proteasomes from *T. acidophilum* and *S. cerevisiae*. The models were determined by analyzing the interactions between PA26 variants and each proteasome in a workflow based on label-free analysis. The ProteOn XPR36 system was employed to characterize the binding kinetics and affinity between the two types of biomolecules. The result shows the ability of the ProteOn XPR36 system to detect dissociation constants at the level of 1 x 10^{-6} s^{-1} and equilibrium constants at the level of 1 pM, which are the typical limits for label-free biosensors in analyzing high-affinity biomolecular interactions.

3.3.2 Thermodynamics and Energetics

Knowing the thermodynamics of biomolecular interactions, in addition to the kinetics, leads to a better understanding of the mechanisms behind binding and helps improve rational drug design. SPR technology is capable of measuring the energetics of a biomolecular interaction by both equilibrium-based and kinetic-based analyses. The typical experiment of thermodynamics involves measuring equilibrium constants under different temperatures and calculating the dissociation constant (K_D) at each temperature. The experimental results are plotted with the Van't Hoff equation to obtain the enthalpy, entropy, and free energy changes of the interaction. When transition state thermodynamics, or energetics, are desired, kinetic constants are measured at different temperatures and plotted with the Eyring equation. It is a useful method for understanding detailed characteristics of biomolecular interactions. Because of the parallel injection feature, the ProteOn XPR36 system allows for high-quality and high-throughput experimental results at different temperatures and greatly enhances the performance of the SPR technology in obtaining thermodynamics of biomolecular interactions. The ProteOn XPR36 system's benefits include:

- Highly efficient thermodynamic analysis workflow for structural biology
- Stable performance and high reproducibility

Published Applications

Characterization of the VWF A1 Domain Epitope for an Antibody

Kulman J (2010). A calcium-dependent immunocapture strategy for enhanced-throughput SPR. Bio-Rad ProteOn Webinar Series.

In order to resolve the binding epitope of VWF (von Willebrand factor) A1 domain against a monoclonal Ab of interest, the interaction was analyzed using the ProteOn XPR36 system (Kulman 2010). A series of A1 mutants were designed and expressed in cell lines and were screened against the antibody using the One-shot Kinetics approach. The measurement was taken over a temperature range to achieve thermodynamic analysis (Figure 3.23). From this experiment, both kinetic constants and transition state thermodynamic parameters were obtained. According to the values of free energy, enthalpy, and entropy changes in the interactions, the contribution of different residues at the potential epitope region was resolved. Compared to conventional biosensors, the high throughput of the ProteOn XPR36 system shortens the experiment time from a month to days.

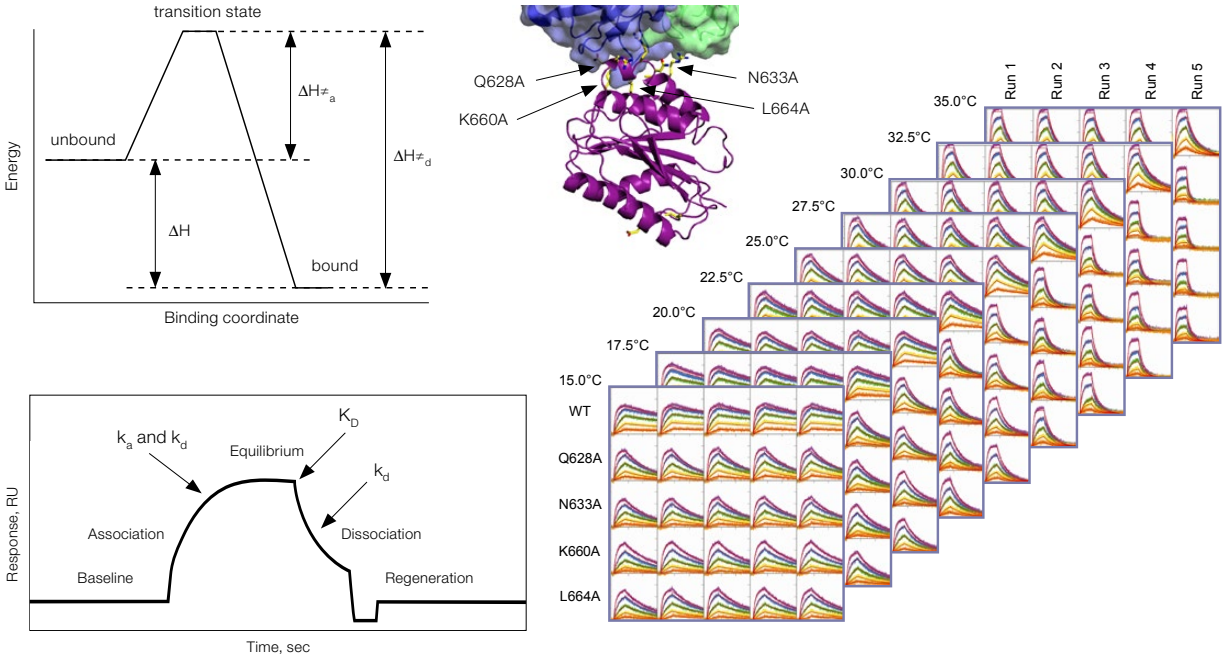


Fig. 3.23. Thermodynamic analysis for epitope mapping in the Ag-Ab interaction.

3.3.3 Histidine-Tagged Protein Analysis

The ProteOn XPR36 system, combined with the HTG and HTE chips, offers the capability to achieve high-quality interaction analysis of histidine-tagged proteins. The HTG and HTE chips feature a novel Tris-NTA technology that provides the binding stability and binding selectivity to histidine-tagged proteins, and surface regenerability of the sensor chips. In the field of label-free biomolecular interaction analysis, this is a significant advance from the traditional approaches, which exhibit considerable baseline drift due to low binding stability. Benefits of HTG and HTE chips include:

- Stability of histidine-tagged protein capture for accurate analysis
- Selectivity for efficient online purification process
- Regeneration capability for low cost

Published Applications

A Wide Range of Applications for the HTG and HTE Sensor Chips

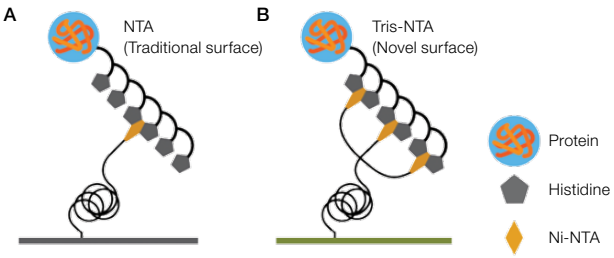
Bronner V et al. (2012). The ProteOn HTE sensor chip: novel surface for stable capture of histidine-tagged proteins for protein-small molecule interaction analysis. Bio-Rad Bulletin 6254.

Luo R et al. (2012). Label-free drug screening against histidine-tagged proteins using novel ProteOn sensor chips. Poster presented at Drug Discovery Chemistry, San Diego, USA, Apr. 2012.

Rabkin E et al. (2012). The ProteOn HTG sensor chip: novel surface for stable capture of histidine-tagged proteins for protein-protein interaction analysis. Bio-Rad Bulletin 6132.

The histidine tag is one of the most widely used tags in protein purification. Recombinant proteins containing the histidine tag are easily captured by an NTA surface. Bio-Rad offers the HTG and HTE chips for capturing histidine-tagged proteins for interaction analysis. The chips employ a novel tris-NTA surface chemistry, which provides higher stability and selectivity in capturing histidine-tagged proteins, compared to the traditional NTA surface chemistry, and allows for surface regeneration. This surface intercalates at three points with a histidine-tagged protein rather than a single point for the NTA surface (Figure 3.24). The HTG chip is ideal for the analysis of protein-protein and protein-peptide interactions, and HTE chip for protein–small molecule interactions (Figure 3.25). The technical notes and the poster describe both protein-protein and protein–small molecule interactions performed using the HTG and HTE chips.

Comparison of NTA and tris-NTA binding to histidine-tagged proteins



Percent of protein that remained bound 5 min after end of injection

	Tris-NTA	NTA
Protein A	100%	97%
Protein A/G	96%	88%
Ubiquitin	92%	45%

Fig. 3.24. Description of tris-NTA technology.

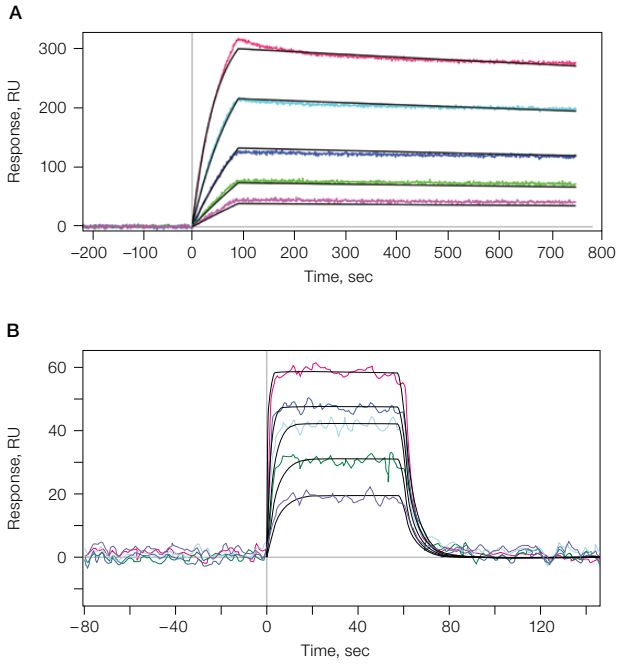


Fig. 3.25. ProteOn HTG and HTE sensor chips. A, sensorgrams of the interaction between the histidine-tagged protein A and IgG, showing the ability of the HTG chip to resolve high-affinity kinetics requiring long dissociation times. Protein A was captured to approximately 60 RU, and human IgG was injected in a twofold dilution series ranging from 100–6.3 nM; **B**, sensorgrams of the interaction between histidine-tagged Erk2 (a MAP kinase) and the inhibitor Purvalanol B (432.9 Da), showing that small molecules can be screened using the HTE chip. Erk2 was captured to approximately 12,800 RU, and Purvalanol B was injected in a threefold dilution series ranging from 50 µM to 0.62 µM. RU, response units.

Characterization of the Interaction of ODC and Az
Cohavi O et al. (2009). Docking of antizyme to ornithine decarboxylase and antizyme inhibitor using experimental mutant and double-mutant cycle data. J Mol Biol 390, 503–515.

This article (Cohavi et al. 2009) uses the ProteOn XPR36 system to analyze the interaction between ornithine decarboxylase (ODC) and a regulatory protein antizyme (Az). The binding sites of ODC on Az were mapped using high-throughput mutagenesis and computational docking. Double-mutant cycle (DMC) analysis between residues on Az and ODC was used to obtain further insights on the structure and function of the complexes.

3.3.4 Nucleic Acid Interaction Analysis

The interactions between proteins and nucleic acids have increasingly drawn attention in the biological research community, as the understanding of these interactions often bridges genomics and proteomics. The ProteOn XPR36 system is capable of analyzing protein-nucleic acid interactions with high performance.

Published Applications

A Wide Range of Applications for the NLC Sensor Chip
Cohen S et al. (2006). Applications of the ProteOn NLC sensor chip: antibody-antigen, DNA-protein, protein-protein interaction analysis. Bio-Rad Bulletin 5449.

This technical note (Cohen et al. 2006) describes the specificity and stability of biotinylated ligands captured to the ProteOn NLC sensor chip. Three common biomolecular interaction models representing antigen-antibody, DNA-protein, and protein-protein interactions were analyzed using the One-shot Kinetics approach. These three interaction types were analyzed in high throughput with the 6 x 6 interaction array (Figure 3.26).

***Trp* repressor and *trp* operator**

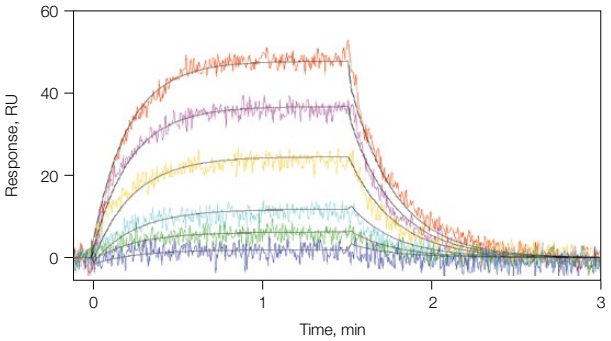


Fig. 3.26. Interaction analysis of an oligonucleotide containing the *trp* operator sequence and the *trp* repressor protein. *Trp* repressor concentrations; 8 nM (–), 6 nM (–), 4 nM (–), 2 nM (–), 1 nM (–), and 0.5 nM (–). RU, response units.

Stephen A et al. (2009). Determining the binding kinetics of HIV-1 nucleocapsid protein to six densities of oligonucleotide using the ProteOn XPR36 protein interaction array system. Bio-Rad Bulletin 5846.

ProteOn XPR36 system is used to analyze the binding kinetics of the HIV-1 nucleocapsid protein (NC) with a short deoxynucleotide $d(TG)_5$ (Stephen et al. 2009). Six different surface densities of $d(TG)_5$ were achieved in the six ligand channels of a sensor chip, and five concentrations of NC were tested in the analyte channels. The low ligand density channels showed excellent sensorgrams (Figure 3.27). The results are consistent with previous work.

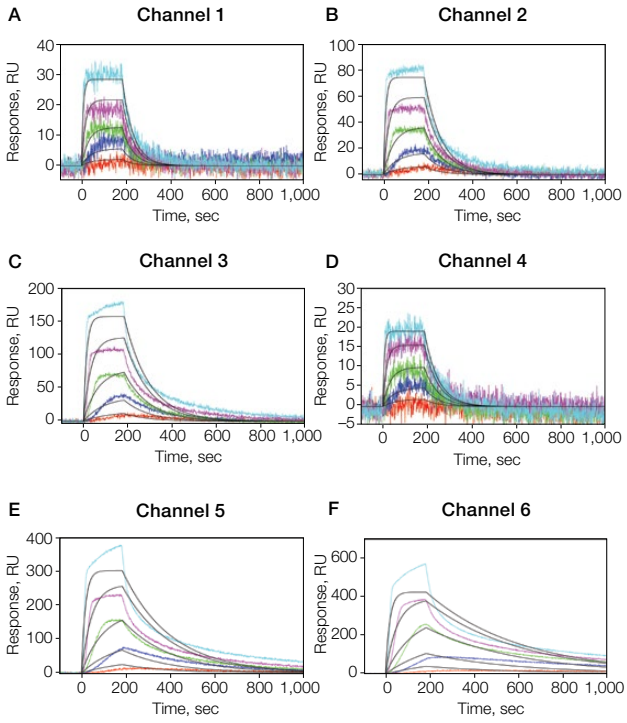


Fig. 3.27. Interaction kinetics of NC binding to different densities of $d(TG)_5$ are compared to the Langmuir 1:1 model. The black trace represents the global fit of the sensorgrams to the 1:1 interaction model. The interactions between six different $d(TG)_5$ ligand densities with five concentrations [300 nM (–); 100 nM (–); 33 nM (–); 11 nM (–); 3.7 nM (–)] of NC were tested. RU, response units.

Interaction Analysis of Pt Squares to G-Quadruplexes
Zheng X et al. (2012). Platinum squares with high selectivity and affinity for human telomeric G-quadruplexes. Chem Commun 48, 7607–7609.

Two new Pt squares with quinoxaline-bridges selectively stabilize human telomeric G-quadruplexes with high binding constants (10^{-7}–10^{-9} M) and an unprecedented binding stoichiometric ratio of Pt square/G-quadruplex (6:1) (Zheng et al. 2012). The binding affinity is measured using the parallel SPR analysis in the ProteOn XPR36 system.

3.3.5 Lipid Membrane and Membrane Protein Analysis

The ProteOn XPR36 system allows for interaction analysis of lipid bilayer membranes or membrane proteins with other biomolecules. Membrane proteins encapsulated in liposomes or lipoparticles may be immobilized to sensor chips to investigate the interactions. In addition, because of its flexibility for experiment design, the ProteOn XPR36 system is used as a platform for solution-based membrane protein research with soluble recombinant proteins. The benefits include:

- Hydrophilic surface chemistry allowing for high performance for capturing lipid assemblies
- Hydrophilic surface chemistry allowing for easy regeneration
- Real-time referencing for reliable experimental results

Published Applications

Applications of Liposome Capturing Kit and GLC Lipid Kit

Luo R et al. (2012). Novel lipid/membrane protein application kits for label-free biomolecular interaction analysis. Poster presented at PEGS, Boston, USA, May 2012.

The ProteOn liposome capturing kit and the ProteOn GLC lipid kit are the novel lipid/membrane protein application kits designed for capturing lipid assemblies such as liposomes for lipid-protein, lipid–small molecule, and membrane protein-protein interaction analysis (Luo et al. 2012). The kits have been used in a variety of applications that characterize lipid-based biomolecular interactions, including (1) antibody interacting with small molecules on liposomes, (2) small molecule drugs partitioning between aqueous solution and lipid bilayer, and (3) membrane disruptive peptides interacting with liposomes (Figure 3.28). The poster shows the value of using these products for lipid membrane and membrane protein research.

Edri M et al. (2013). Novel liposome-capture surface chemistries to analyze drug-lipid interaction using the ProteOn XPR36 system. Bio-Rad Bulletin 6449.

Luo R et al. (2013). Novel Liposome-Capture Surface Chemistries to Analyze Drug-Lipid Interaction Using the ProteOnTM XPR36 System. Poster presented at AAPS, San Antonio, USA, Nov. 2013.

The ProteOn liposome capturing kit used within the ProteOn XPR36 system provides a novel hydrophilic surface chemistry that allows for advanced applications such as drug-lipid interaction analysis (Edri et al. 2013). This technical note and the poster show that it is possible to establish an evaluation tool using this kit for small molecule drug delivery, including measuring the properties of drugs and evaluating drug carrier systems (Figure 3.29).

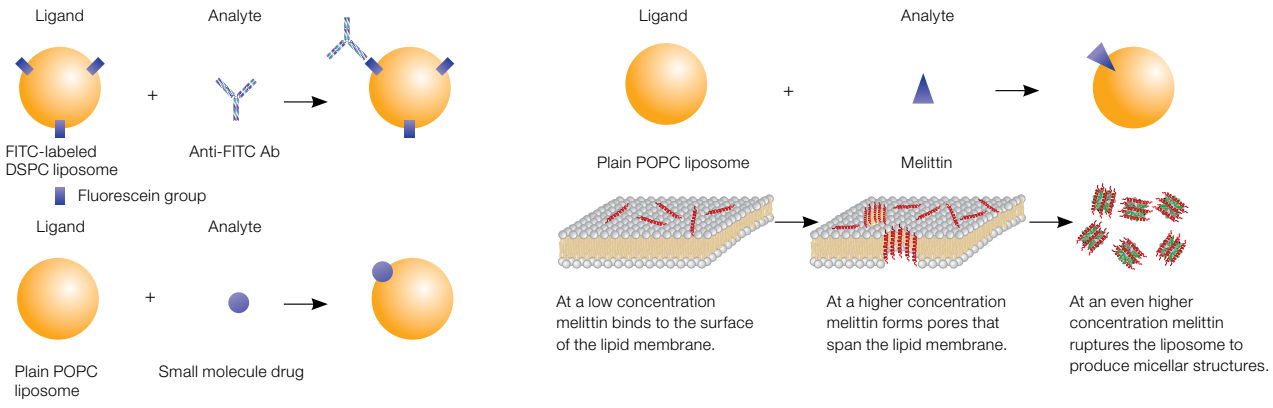


Fig. 3.28. Use the lipid/membrane protein application kits to analyze liposome-protein, liposome-peptide and liposome–small molecule interactions.

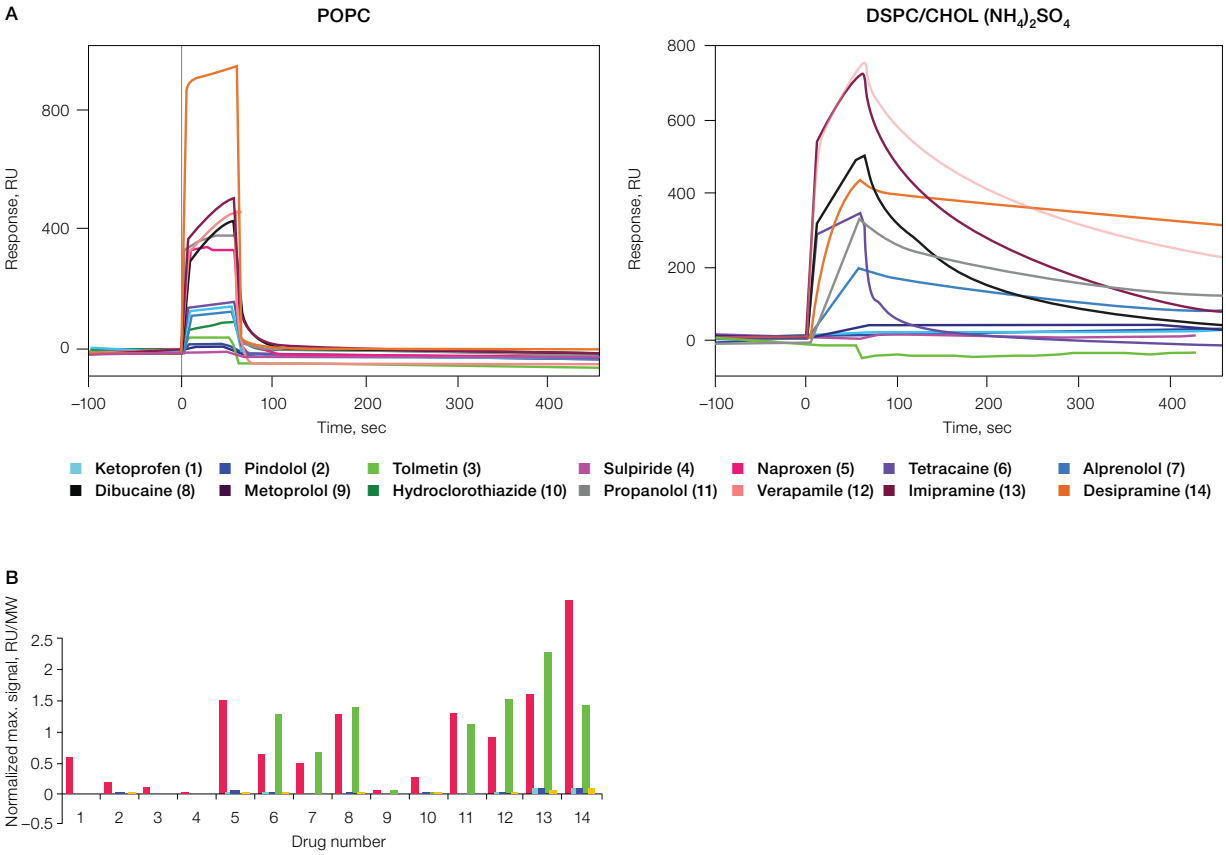


Fig. 3.29. The results of the screening of 14 small molecule drugs against five liposomes using the 6 x 6 configuration in the ProteOn XPR36 system. A, sensorgrams obtained from the ligand channels of POPC and DSPC/CHOL $(NH_4)_2SO_4$. Weak or no responses were observed in the other ligand channels, indicating no interactions between the small molecule drugs and the other three liposomes; **B**, normalized maximum signals of the interactions of the drugs and the liposomes. Responses were normalized by dividing by the compound's MW. Strong responses were observed when using POPC liposomes and ammonium sulfate gradient DSPC/CHOL $(NH_4)_2SO_4$ liposomes. POPC (■); DSPC/CHOL (67/33) (■); DSPC/CHOL (55/45) (■); DSPC/CHOL $(NH_4)_2SO_4$ (■); DSPC/CHOL/PEG (50/45/5) (■). RU, response units; MW, molecular weight.

Membrane Protein Analysis Using Lipoparticles

Willis S (2009). ProteOn XPR36 and lipoparticle technology – A powerful combination for screening antibody therapeutics against membrane proteins. Bio-Rad ProteOn Webinar Series.

This webinar (Willis 2009) provides an overview of the Integral Molecular lipoparticle technology. Lipoparticles are virus-like particles that contain high levels of the protein of interest. The protein is correctly folded, homogenous, stable, and highly active. This offers a unique solution for those researchers looking to work with membrane proteins. Combining this technology with the ProteOn XPR36 system offers an easy-to-use robust approach to screen antibody therapeutics against membrane proteins in a high-throughput manner.

Bronner V et al. (2011). Highly efficient lipoparticle capture and SPR binding kinetics of a membrane protein using the ProteOn XPR36 protein interaction array system. Bio-Rad Bulletin 6161.

The technical note (Bronner et al. 2011) provides an introduction to the novel liposome immobilization technology called the MemLAYER technology. Immobilization protocols for liposomes and lipoparticles using this technology in the ProteOn XPR36 system were shown. The method of membrane protein-antibody interaction analysis is investigated by comparing other lipoparticle immobilization approaches. The screening of drug candidates against malaria proteins using the novel SPR-based approach is discussed. MemLAYER technology is employed in the ProteOn liposome capturing kit (Figure 3.30).

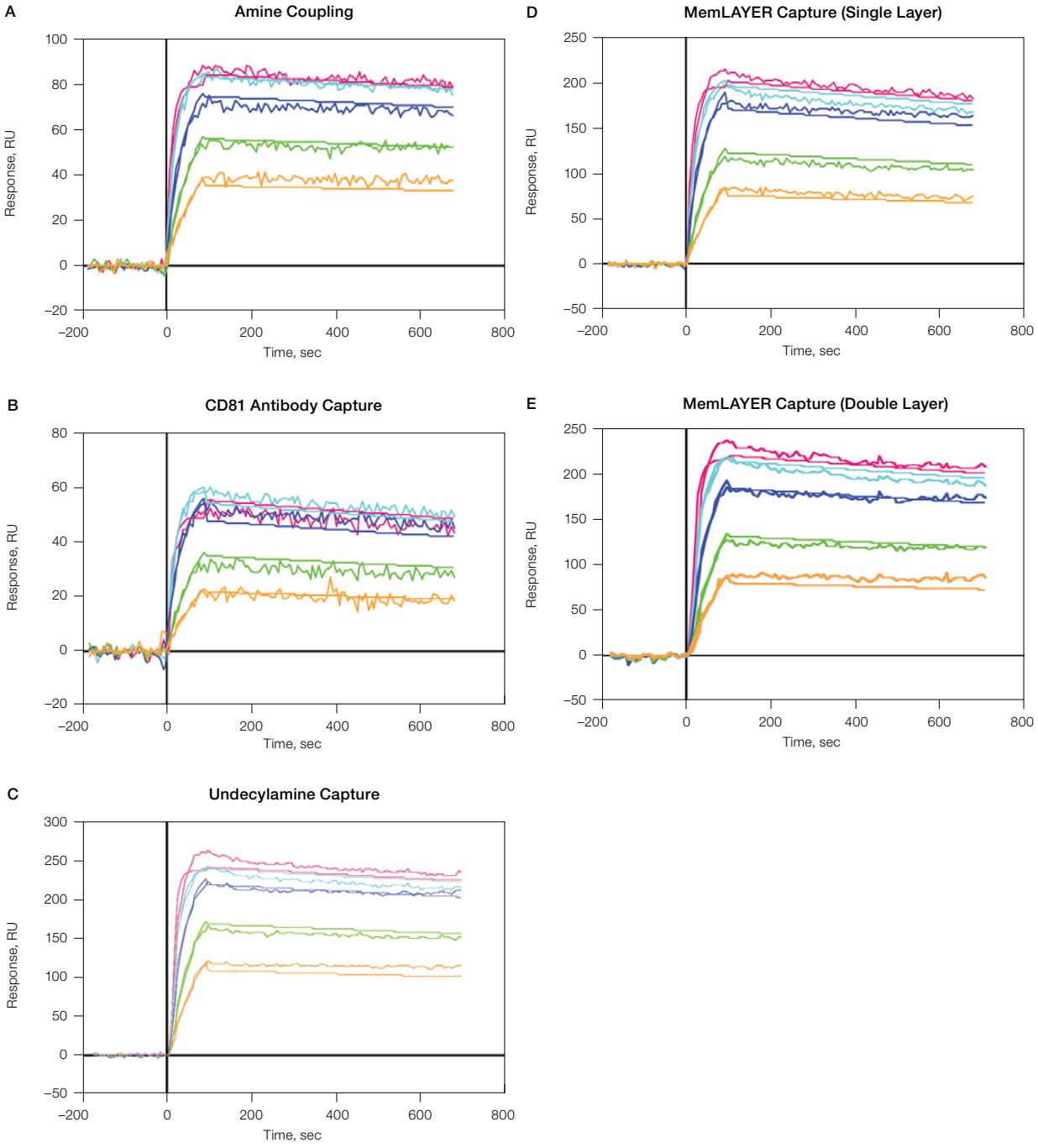


Fig. 3.30. Kinetic analysis for comparing the different lipoparticle immobilization approaches. CXCR4 lipoparticles were immobilized on GLM using the four described methods, followed by One-shot Kinetics injection of CXCR4 antibody. **A** through **E** show the sensorgrams for the five analyte concentrations with the overlaid 1:1 model fit. 30 nM (■); 15 nM (■); 7.5 nM (■); 3.75 nM (■); 1.875 nM (■). RU, response units.

Identification of Membrane Protein Interactions Through Domain Screening

Jiang L et al. (2010). Identification of leucocyte surface protein interactions by high-throughput screening with multivalent reagents. Immunology 129, 55–61.

This article (Jiang et al. 2010) describes a method to screen the interactions between membrane protein extracellular domains. It uses the 36-ligand array approach of the ProteOn XPR36 system to attain high-throughput results. Approximately 36 unique domains were immobilized to a sensor chip surface and qualitative binding results were determined against the same 36 domains injected as analytes across the surface. A few interactions were discovered between different membrane protein extracellular domains and specific research on the membrane proteins containing these extracellular domains.

3.3.6 Cell-Antibody Interaction Analysis

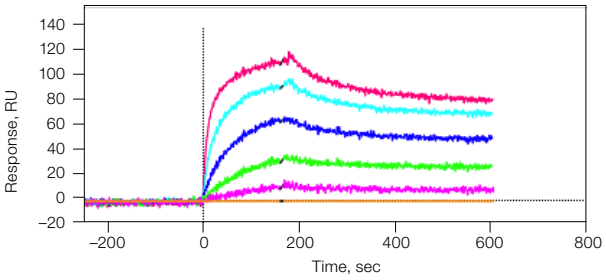
A new application of the ProteOn XPR36 system is the investigation of whole cell-protein interactions. Mammalian or bacterial cells are captured on sensor chips immobilized with cell-specific antibodies, allowing for the interaction of these cells with different proteins.

Published Applications

Analysis of Cell-Antibody Interactions

Adachi S et al. (2011). Interaction analysis of cell-protein using surface plasmon resonance (SPR) method. Medical Science Digest (Japanese), 37, 33–38.

This article (Adachi et al. 2011) employs the ProteOn XPR36 system to identify the binding of bacterial cells to a sensor chip surface. *Staphylococcus aureus* bacteria, expressing a high level of protein A on their surfaces, were prepared in a single cell suspension. The cells have a diameter of approximately 1 µm. An IgG surface was used to successfully capture bacterial cells from the suspension through the high-affinity binding between Protein A and the IgG Fc fragment. In addition, two experiments were performed to show the possibility of analyzing non-adherent mammalian cells using the same method (Figure 3.31).



Ligand: rabbit IgG
Analyte: Staphylococcus aureus
Dilution ratio: A1: x10; A2: x30; A3: x90; A4: x270; A5: x810
Flow rate: 100 ul/min

Fig. 3.31. Kinetic analysis of the IgG Fc fragment — *Staphylococcus aureus* interaction.

3.3.7 Regenerable Biotin-Capture Surface

Ligand capture method is a strategy often employed in an SPR experiment to increase the data quality and the reusability of sensor chips. To apply the ligand capture method, it is required that an affinity tag is linked to the ligand in order to facilitate the binding between the ligand and the capture reagent. Widely used affinity tags include biotin, histidine-tag (polyhistidines), etc. Biotin is particularly preferred in some cases due to the ease of biotinylation to biomolecules and its high affinity to the avidin family proteins, such as avidin, streptavidin, and NeutrAvidin. However, the high affinity between biotin and streptavidin also results in difficulty of surface regeneration. A new method of biotin-based ligand capture with surface regeneration capability is possible. This new method takes two affinity tags, biotin and histidine tag, to build a multi-layer ligand immobilization configuration. It incorporates the advantages of both affinity tags: the regeneration capability of the histidine tag surface chemistry and the high-affinity biotin surface chemistry.

Published Applications

Proof of Principle for Regenerable Biotin-Capture Surface

Zhu M et al. (2013). A novel biotinylated ligand-capture method with surface regeneration capability for label-free biomolecular interaction analysis. BioRadiations Feb. 2013.

This article (Zhu et al. 2013) describes the workflow of using both HTG and HTE chips to achieve regenerable biotin-capture surface. As the proof of principle, two steps were implemented in an SPR experiment: (1) histidine-tagged streptavidin was captured to the chip surface through the interaction between the histidine tag and the activated tris-NTA on the chip surface, and (2) a biotinylated ligand was captured to the streptavidin to prepare the ligand surface for interaction analysis. In this approach, the histidine-tagged streptavidin is the primary capture reagent and the activated tris-NTA is the secondary (Figure 3.32). When it comes to surface regeneration, a chelate such as EDTA is typically used to break the binding between histidine tag and tris-NTA, and the original surface with only tris-NTA is restored. Excellent results were shown for this approach.

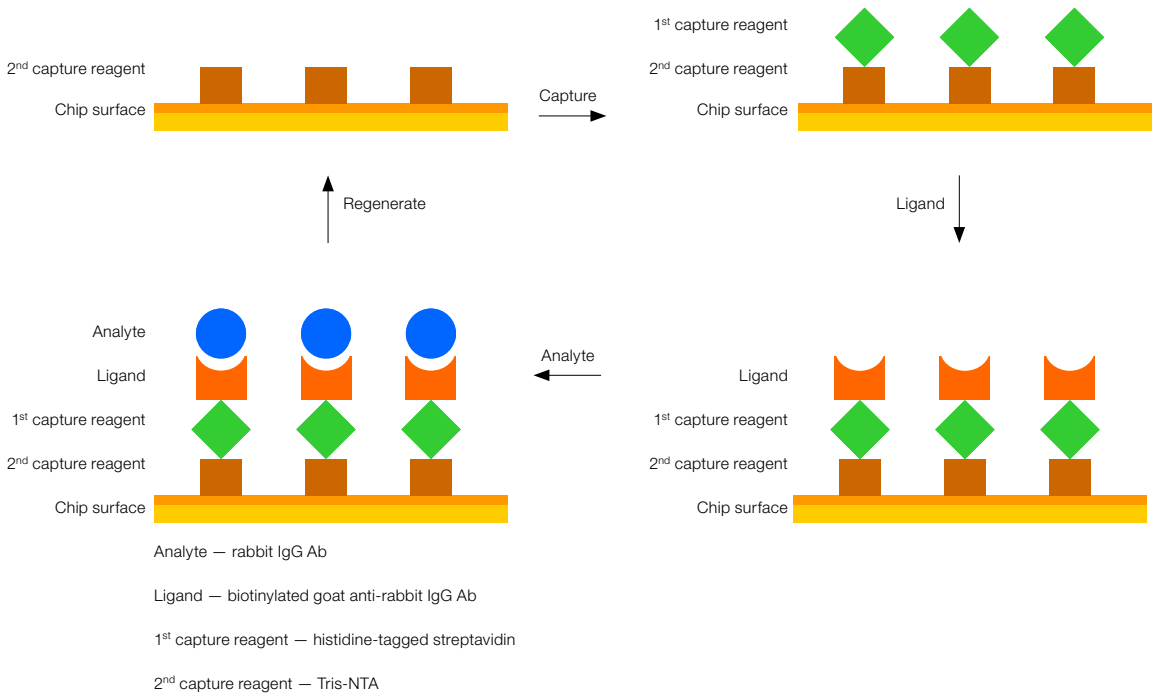


Fig. 3.32. Workflow of the regenerable biotin-capture surface based on HTG and HTE chips.

3.3.8 SPR-MS Analysis

SPR-matrix-assisted laser desorption/ionization (MALDI)-MS coupling has been a constant demand in biology. It combines the advantages of SPR and MS for analyte characterization and identification in a single experiment. Traditionally the analytes are collected by elution from the sensor chip surface after kinetic analysis. However, it is possible to directly detect surface-captured analytes on an SPR sensor chip with no elution steps, since an SPR sensor chip is coated with a conductive gold layer that makes it highly adaptable to MALDI-MS. A new method is possible for SPR-MALDI-MS coupling using the ProteOn XPR36 system that allows direct detection from the chip surface.

Published Applications

Proof of Concept for SPR-MS Analysis

Luo R et al. (2012). Analyzing binding kinetics with surface plasmon resonance complemented with direct mass spectrometry on the same sensor chip. BioRadiations Aug. 2012.

Roth S et al. (2011). Secondary analysis of SPR based arrays by direct use in MALDI time of flight mass spectrometer. Poster presented at HUPO World Congress, Geneva, Switzerland, Sep. 2011.

This article and poster describe the proof of concepts of SPR-MS analysis using the ProteOn XPR36 system. In the experiment, β-amyloid peptide fragments were first analyzed and captured by SPR and subsequently analyzed by MALDI-MS to identify the mass of each individual peptide fragment. For kinetic characterization, One-shot Kinetic analysis of the interaction of 6E10 Ab and β-amyloid 1–40 was performed. The surface was regenerated with phosphoric acid, and reloaded with β-amyloid 1–40 (80 nM) for MS analysis. The chip was rinsed in water and mounted to a customized adaptor, and then MALDI-MS analysis was carried out in a mass spectrometer (Figure 3.33).

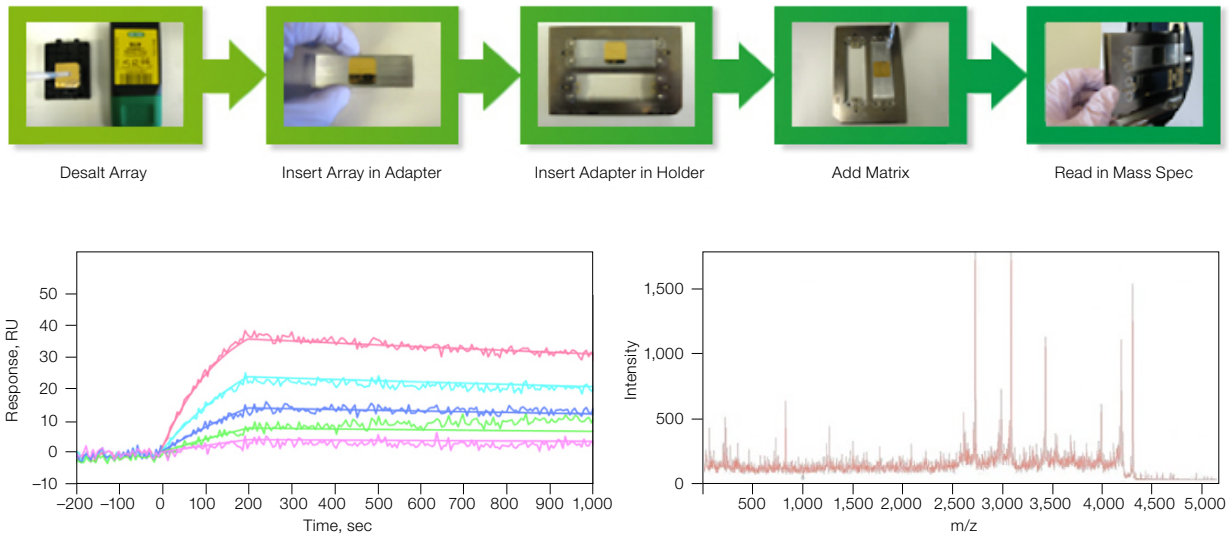


Fig. 3.33. SPR-MS analysis using direct MALDI-MS analysis from the chip surface. RU, response units.

3.4 Biological Assays

Due to the vast array of applications for SPR, label-free interaction protocols for the ProteOn XPR36 must be tailored to specific experimental needs to obtain high-quality SPR data. With the deposition of six or more ligands on one sensor chip, the ProteOn XPR36 system's unique 6 × 6 interaction array allows for flexibility in the design of biomolecular interaction assays, streamlining assay design. Interrogating these different ligands with six separate analytes facilitates experimental optimization by enabling the real-time detection of up to 36 different biomolecular interaction events simultaneously.

3.4.1 Assay Design and Optimization

Assay design is essential for obtaining high quality SPR results. Selecting and optimizing the most suitable experimental conditions is of utmost importance for assay accuracy and reproducibility. The novel 6 x 6 interaction array of the ProteOn XPR36 system provides the versatility for many different types of experiments on a single platform at high throughput. This advantage allows for simultaneous investigation of multiple experiment conditions of an interaction, and also rapid reproducibility testing. Because of the unique advantages in assay design, the ProteOn XPR36 system provides the highest efficiency and accuracy compared to other SPR platforms available. Advantages include:

- Rapid screening for reagents
- Flexible assay configuration
- Multiple surfaces for different assays
- Unattended running for assay validation

Typical assay optimization involves screening different experimental conditions for ligand immobilization and analyte injection, including ligand density, immobilization pH, analyte concentration, ionic strength, choice of additives, etc. The combination of different parameters results in a large matrix of experimental conditions for optimization. Utilizing the novel 6 x 6 interaction array, the ProteOn XPR36 system has the power to achieve an entire optimization experiment in a short period of time and provides the highest efficiency and accuracy compared to other SPR platforms available. Optimization advantages include:

- 6 x 6 experiment conditions in an injection
- Efficient data analysis by the software

Published Applications

Design and Optimization of Antibody Analysis Assays

Bronner V et al. (2009). Rapid screening and selection of optimal antibody capture agents using the ProteOn XPR36 protein interaction array system. Bio-Rad Bulletin 5820.

This technical note (Bronner et al. 2009) describes how the One-shot Kinetics approach was used to rapidly screen the binding of four antibody-binding proteins and seven types of antibody targets. The selection of antibody-binding proteins that provide the optimal binding characteristics for the capture of each antibody type was achieved rapidly in the ProteOn XPR36 system. The One-shot Kinetics approach allows for the analysis of multiple experimental conditions in a single experiment (Figure 3.34).

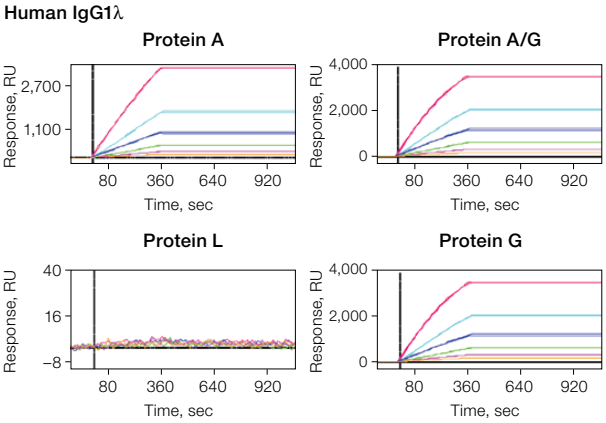


Fig. 3.34. Screening of human IgG1λ to the immunoglobulin-binding proteins. RU, response units.

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

This technical note (Bronner et al. 2006) describes the rapid and detailed characterization of four Ag-Ab interactions by the ProteOn XPR36 system to fully exploit the 6 x 6 interaction array. Four different antibody targets and a negative control were immobilized in five ligand channels, leaving one ligand channel blank to serve as reference (Figure 3.35). A concentration series of each antigen was injected in six analyte channels. Ligand surface regeneration was performed before each new analyte injection.

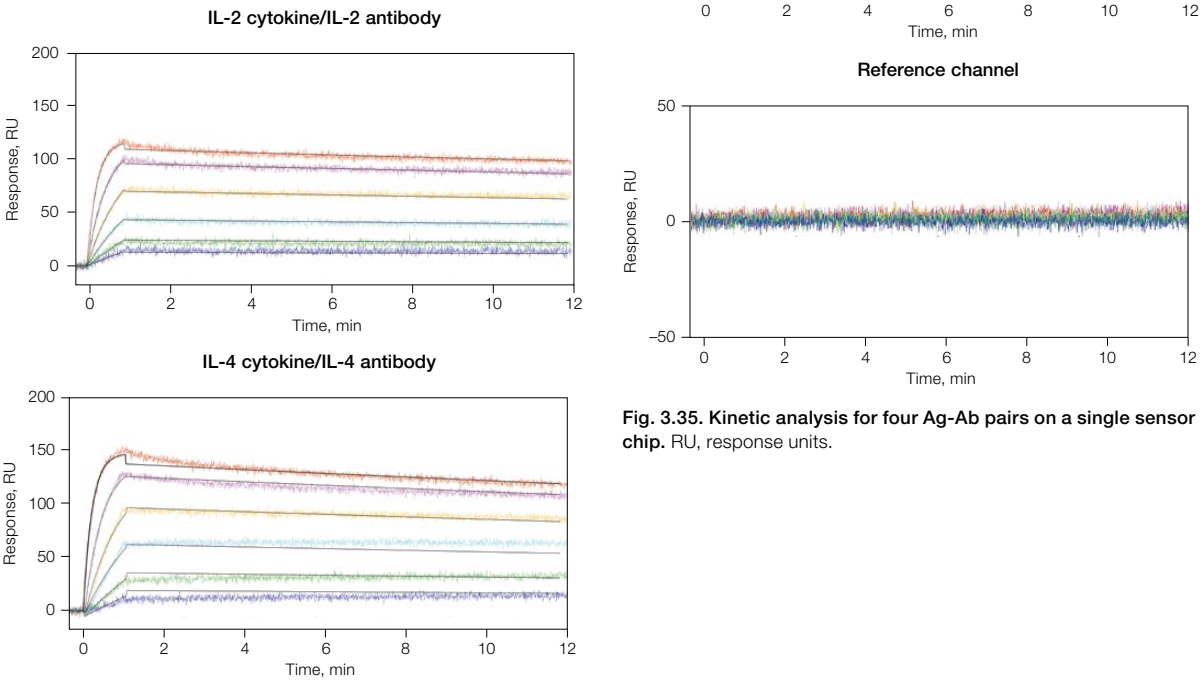


Fig. 3.35. Kinetic analysis for four Ag-Ab pairs on a single sensor chip. RU, response units.

3.4.2 Biosimilar Assessment

Antibody drugs have constituted the major part of biotherapeutics. Biosimilars are generic versions of existing approved antibody drugs. Determination of the Ab-Fc receptor binding affinity of biosimilars is essential for understanding the mechanism by which the drug functions and the similarity between biosimilars and the corresponding original drugs. The N-linked oligosaccharide side chain of the Fc region varies in different cell lines and even in different batches, which compels biopharmaceutical manufacturers to analyze Ab-Fc receptor binding affinity during the discovery and manufacturing phases (Figure 3.36). The ProteOn XPR36 system has been used as an essential tool for biosimilar assessment in Ab-Fc receptor binding affinity analysis.

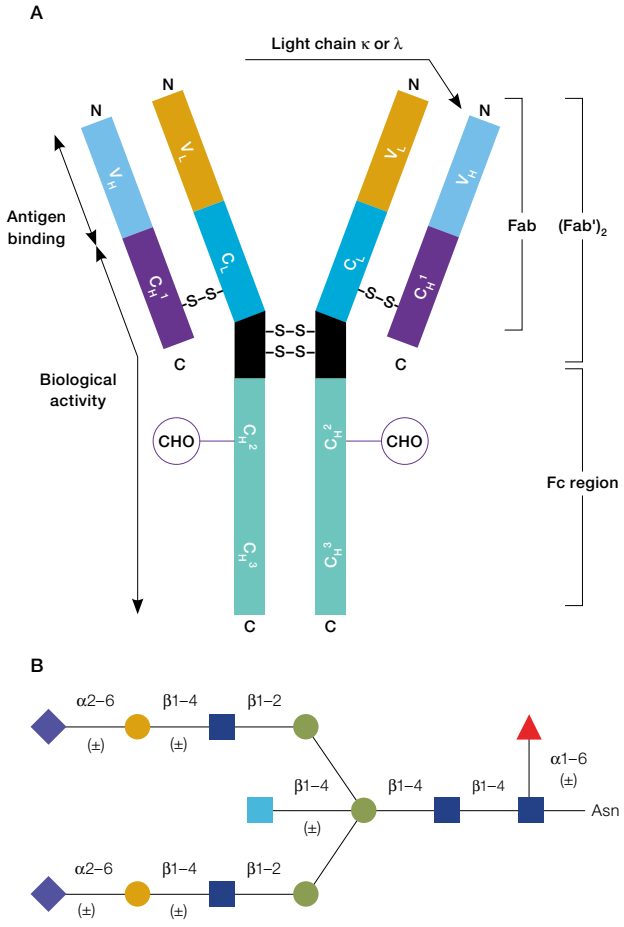


Fig. 3.36. Human antibodies and their variable oligosaccharide side chains. A, structure of a human antibody, depicting the amino-terminal (N), carboxy-terminal (C), heavy and light chain variable domains (V_H and V_L, respectively), heavy and light chain constant domains ($C_H^{1/2/3}$ and C_L, respectively), disulfide bonds (S-S), and oligosaccharide side chains (CHO); **B**, example of an oligosaccharide side chain, with the following components: (■) N-acetylglucosamine; (■) bisecting N-acetylglucosamine; (●) mannose; (●) galactose; (◆) sialic acid; (▲) fucose.

3.5 Biomedical Applications

Recently, the uses of the ProteOn XPR36 system have extended to a number of biomedical applications, which were not typically performed by the SPR community, including vaccine evaluation and clinical diagnostics. These new applications have benefited from the usability and experimental convenience of the ProteOn XPR36 system. They have also broadened the scope of SPR technology.

3.5.1 Vaccine Characterization

The compatibility with clinical samples enables the ProteOn XPR36 system to provide both concentration analysis and kinetic profiling of active components in clinical samples. Therefore, it is an efficient tool in the evaluation of vaccines for immunogenicity analysis and potency determination.

Published Applications

Vaccine Immunogenicity Analysis

Khurana S et al. (2010). Properly folded bacterially expressed H1N1 hemagglutinin globular head and ectodomain vaccines protect ferrets against H1N1 pandemic influenza virus. PLoS ONE 5, e11548.

Khurana S et al. (2010). Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus. Sci Transl Med 2, 15ra5.

These articles describe how SPR technology provided by the ProteOn system was used to facilitate vaccine production against pandemic diseases. For example, in the first article, the H1N1 A/California/07/2009 virus was the target. A recombinant protein approach was used to achieve rapid large-scale production of vaccines. The ProteOn system was used as a quality control tool. H1N1-HA (1-330) and H1N1-HA (1-480) were expressed in *E. coli* under controlled redox refolding conditions. H1N1-HA0 was a mammalian cell-derived recombinant full length H1N1-HA virus. The ProteOn system was used to verify the expression of conformational 'native' antigenic epitopes. The articles illustrate a promising workflow for the rapid large-scale production of vaccines.

Vaccine Potency Determination

Khurana S et al. (2014) Novel antibody-independent receptor-binding SPR-based assay for rapid measurement of influenza vaccine potency. Vaccine 32, 2188–2197.

An antibody-independent, simple, high-throughput receptor-binding SPR-based potency assay is proposed using the ProteOn XPR36 system. The assay measures the binding between influenza vaccine strains in sample flow and synthetic glycans immobilized on the surface of a chip. The active forms of hemagglutinin in vaccine samples are quantified by the initial binding slopes and thus vaccine potency is determined. The advantages of this SPR-based potency assay are: (1) it does not require any reference antiserum sample and (2) it can be used for rapid hemagglutinin quantitation and vaccine release.

The ProteOn XPR36 system shows high performance in the potency assay and allows the testing of multivalent vaccines. Excellent concordance is shown between the SPR-based potency assay and the standard single-radial immunodiffusion (SRID) assay (Figure 3.37).

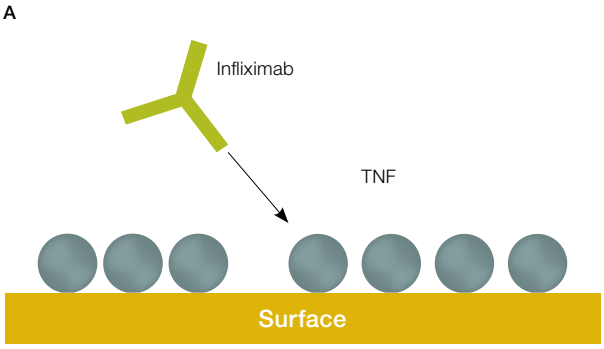
3.5.2 Clinical Diagnostics

The compatibility with clinical samples extends the applications of the ProteOn system to clinical diagnostics. It has been used as an efficient tool in active serum component quantitation.

Published Applications

Antibody Drug Companion Diagnostics

Thoren K (2014). SPR as a new technology in clinical research. Bio-Rad ProteOn Webinar 2014 Series.



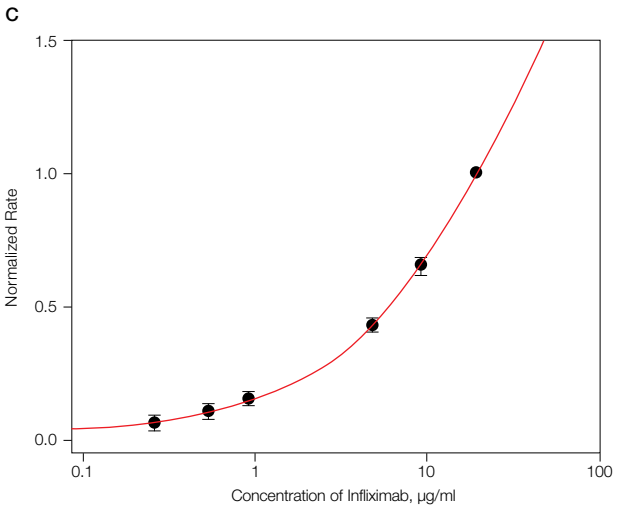
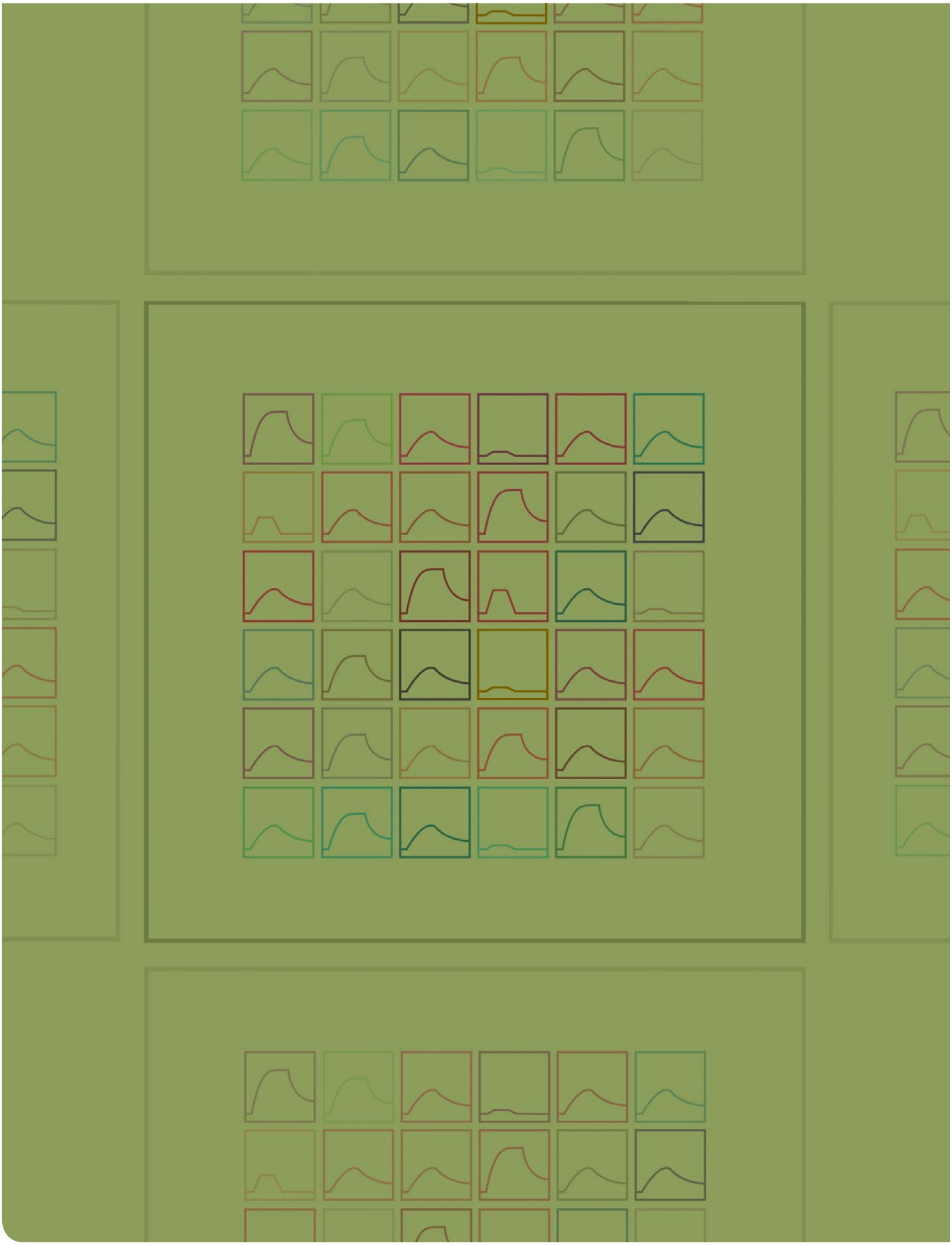


Fig. 3.37. Quantitation of infliximab in serum samples. A, illustration of the SPR surface chemistry; **B**, quantitation based on the initial rate of the ligand-analyte interaction, (■) 20 µg/ml; (■) 10 µg/ml; (■) 5 µg/ml; (■) 1 µg/ml; (■) 0.6 µg/ml; (■) 0.3 µg/ml; **C**, the initial rate obtained from standard analyte samples is plotted against analyte concentrations to form a standard curve.

This webinar highlighted how the ProteOn XPR36 system was used to quantitatively measure the serum levels of the TNFα inhibitor drug, infliximab. Infliximab is a chimeric monoclonal antibody that targets TNFα and is used to treat a variety of chronic autoimmune disorders. Monitoring serum infliximab concentrations is important in guiding management, especially when a patient is not responding well to treatment. Infliximab concentrations can determine if loss of efficacy is due to an inadequate dose or to the development of anti-infliximab antibodies. Currently, quantitative infliximab testing is costly. Using the ProteOn XPR36 system, a quantitative assay for infliximab in serum was developed, and in general, the practicality of using SPR in the clinical lab was explored.



CHAPTER 4
Experimental Design

A surface plasmon resonance (SPR) experiment, designed to investigate biomolecular interactions, incorporates an entire workflow including data acquisition, data processing, and data analysis. High-quality SPR results can be obtained when all the steps in this workflow are designed and executed appropriately.

4.1 Introduction to SPR Experimental Design

An SPR experiment to investigate biomolecular interactions incorporates an entire workflow including data acquisition, data processing, and data analysis. High-quality SPR results can be obtained when all the steps in this workflow are designed and executed appropriately. The workflow should be optimized to achieve this goal. This chapter introduces the SPR workflow and provides optimization approaches to facilitate high-quality SPR results.

Four steps are typically performed to complete an SPR experiment: ligand immobilization, analyte injection, data processing, and data analysis, as shown in Figure 4.1. The ligand immobilization step refers to the immobilization of a ligand (the first interaction partner) onto a sensing surface, and the analyte injection step refers to the injection of an analyte (the second interaction partner) in a flow to interact with the ligand. These two steps are the data-generating process in an SPR experiment. Thus, together they are termed data acquisition. The data collected are presented as a time trace to form a sensorgram. Data acquisition, processing, and analysis form the data flow leading to SPR results.

Data processing includes sensorgram processing and sensorgram referencing, where referencing is the main task. Data analysis includes parameter setting followed by sensorgram fitting (for kinetic analysis) or value plotting (for equilibrium analysis and concentration analysis) to yield SPR results.

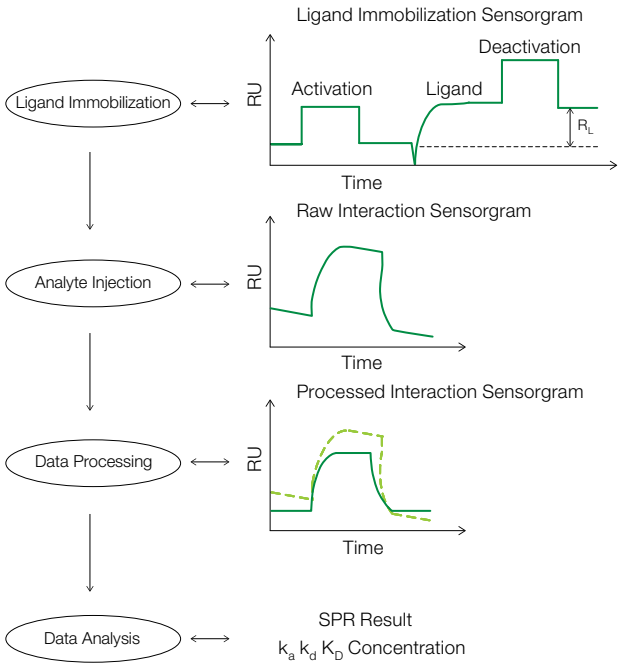


Fig. 4.1. SPR workflow for biomolecular interaction analysis. RU, response units.

In order to provide a user-friendly guide for the use of the ProteOn XPR36 system, the steps of data processing and data analysis using ProteOn Manager software are outlined in section 4.7, and the options for exporting SPR results for presentation are described in section 4.8.

4.1.1 ProteOn™ XPR36 System

The ProteOn XPR36 system is an SPR biosensor platform consisting of the ProteOn XPR36 instrument and ProteOn Manager™ software. The system features a novel 6 × 6 experimental configuration for multiplexed interactions among multiple targets and analytes. This unique, patented design facilitates the simultaneous investigation of 36 different interactions using the One-shot Kinetics™ approach. The ProteOn XPR36 system facilitates the generation of high-quality SPR results by providing versatility in experimental design, reproducible instrument performance, comprehensive referencing options, and a powerful software user interface. In addition, this platform allows for high-throughput sample processing. Thus, the ProteOn XPR36 system is positioned as an optimal SPR biosensor platform for high data quality and cost-effective experiments.

4.1.2 Checklist of Good Publication Standards

Because implementing biomolecular interaction analysis with SPR biosensors incorporates a multiple-step workflow, all the factors of data acquisition, processing, and analysis should be considered upfront in experimental design. An excellent resource regarding the factors to consider is the checklist of good publication standards developed by the key opinion leaders in the SPR community. These standards help present SPR results in a clear and organized manner for publications, facilitating effective information sharing among researchers using SPR biosensors. This checklist, The Bare Minimum Requirements for an Article Describing Optical Biosensor Experiments (TBMRFAADOBE), is presented here (Rich and Myszka 2011). We highly recommend consulting this checklist prior to submitting SPR results for publication.

TBMRFAADOBE

- Instrument used in analysis
- Identity, source, MW of ligand and analyte
- Surface type
- Immobilization condition
- Ligand density
- Experimental buffers
- Experimental temperatures
- Analyte concentrations
- Regeneration condition
- Figure of binding responses with fit
- Overlay of replicate analyses
- Model used to fit the data
- Binding constants with standard errors

Reference

Rich, RL and Myszka DG (2011). Survey of the 2009 commercial optical biosensor literature. J Mol Recognit 24, 892–914.

4.2 Guide to Ligand Immobilization on the ProteOn XPR36 System

SPR has revolutionized the study of biomolecular interaction by providing a platform that does not require the ligand or analyte to be labeled. SPR measures the interaction between a ligand immobilized to the surface of a sensor chip and an analyte in solution. This measurement takes place in real time, providing kinetic, equilibrium, and concentration data. Performing interaction analysis on an active and stable ligand surface is key to generating robust data. The ProteOn XPR36 system is a multiplexed SPR instrument that utilizes novel fluidics to monitor the interaction of up to six ligands and six analytes. This allows for the simultaneous study of up to 36 interactions on the surface of the sensor chip, greatly increasing experimental throughput and reducing assay development time.

In the simplest SPR experiment using the ProteOn XPR36 system, a ligand is covalently immobilized to the surface of the sensor chip and interacts with an analyte present in the running buffer that flows over the surface of the sensor chip. This is known as direct immobilization.

In another commonly used method, a biomolecule is used to capture the ligand prior to analyte interaction. In this case the ligand is not covalently immobilized on the chip surface but is captured through biomolecular interactions. The advantages of ligand capture are as follows:

- Creates a homogenous ligand surface (well-defined orientation)
- Purifies ligand on the chip surface
- Allows regeneration of the ligand surface

Both methods have different advantages, depending on the type of interaction analysis that is being carried out. The major steps of each method are discussed in this guide.

Part 1: Direct Immobilization of Ligand

Methods of direct immobilization include amine coupling, thiol coupling, and aldehyde coupling. While all these methods are applicable on the general-use GLC, GLM, and GLH chips, the amine coupling method is the most typical in SPR experiments. The ProteOn amine coupling kit contains all the reagents needed for amine coupling of proteins or peptides to the sensor chip.

Major Steps for Covalent Immobilization

1. Conditioning of sensor chip.
2. Immobilization of ligand (activation, immobilization, and deactivation).
3. Stabilization of ligand surface.

4.2.1 Conditioning

Conditioning is recommended for new sensor chips and can generally improve data quality by cleaning the new chip surface, encouraging rapid stabilization of the baseline prior to the start of the experiment. Sensor chip conditioning is optional. Conditioning is performed following the sensor chip initialization process using ProteOn regeneration solutions (refer to Chapter 5).

4.2.2 Activation

In this step reactive groups are formed on the sensor chip surface. The ligand of interest, such as a protein, is then attracted to the surface and binds through amine coupling. Any primary amine within a protein sequence can bind (lysine residues and the N-terminus).

To create these reactive groups an activation solution is applied to the surface. This activation solution consists of an equivolume mixture of two reagents, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS). These two reagents are part of the ProteOn amine coupling kit and are prepared by addition of 7.5 ml water to each reagent bottle to make 400 mM EDC and 100 mM sulfo-NHS, which are stored at –20°C until needed. It is important to make this mixture fresh every time, as it has a half life of 30–60 min and should therefore be used immediately. After thawing and mixing, you may dilute the equivolume activation solution prior to use, depending on which application you are working with.

Chemistry

After addition of EDC and sulfo-NHS to the chip, the carboxyl groups react and become sulfo-NHS esters. During the ligand injection step, the ligand preferentially binds to the esters and is amine-coupled to the chip surface.

4.2.3 Immobilization

Many factors affect ligand immobilization, including chip type, level of surface activation, ligand concentration, size, and injection parameters such as contact time, injection flow rate, and electrostatic attraction of the ligand to the surface. Electrostatic attraction is one of the most important factors because if the ligand is not attracted to the surface, there will be very little immobilization.

Optimizing Immobilization Conditions

After amine coupling, the sensor chip surface will have an overall negative charge; the ligand therefore must have an overall positive charge. This is achieved by determining the optimal immobilization buffer. Since the ProteOn XPR36 system has six ligand channels, you can easily test multiple immobilization conditions (immobilization buffers of different pH) to determine which gives the highest level of immobilization. For example, BSA has a pI of approximately 5.5. To have a positive charge the protein must be dissolved in a buffer of pH less than 5.5. Therefore, one might wish to try a series of buffers with a pH of 5.5, 5.0, 4.5, and 4.0 and monitor which results in the highest level of immobilization. Care must be taken to ensure that the immobilization conditions used result in an immobilized ligand that retains its activity. When using buffers of extreme pH, the ligand may be denatured or unfolded and therefore lose its activity (see Table 4.1). Detergents may also be added into the immobilization buffer but salt should be kept to a minimum, just enough to keep the ligand soluble.

Ligand Injection Parameters

Determining the ideal ligand injection parameters is important. Flow rate and contact time can have significant effects on immobilization. Default injection parameters are 30 µl/min for 5 min. The low flow rate will help to increase immobilization, as will an increase in the injection/contact time.

Ligand Conditions

The concentration of the ligand will also affect the total amount immobilized. Typically, concentrations of 5–100 µg/ml should be sufficient to attain a good level of immobilization.

The ligand stock buffer should have a high concentration of the ligand so that when it is diluted with the immobilization buffer any salts or other additives present in the stock buffer will also be diluted. A good recommendation for ligand stock buffer is 0.5–1 mg/ml. Avoid (or minimize) any other amine-containing compounds (or any strong nucleophilic groups) such as azide or Tris buffer, as these amines will compete with the ligand amines.

Guidelines for Immobilization Levels

What level of ligand immobilization to use depends on the type of interaction under study. However, “less is more” is a good guide and this is generally followed for kinetic binding measurements. With a high-density surface, mass transport issues and crowding effects may result in altered kinetics (see Table 4.1).

An easy way to help determine which ligand level to use is to calculate the theoretical R_{max} of the interaction to be studied.

Using R_{max} to Determine Ligand Immobilization Levels

The theoretical R_{max} is the maximum analyte response, assuming all of the ligand is active, ligand is 100% pure, and all binding sites are available. When using amine coupling, assume that not all ligand binding sites will be available after immobilization, since this is a random coupling of the ligand to the sensor chip and therefore the ligand is not present in a homogenous orientation at the sensor chip surface (see Figure 4.2).

$$R_{max} = \frac{MW_A}{MW_L} \times R_L \times n$$

R_{max}, maximum theoretical response of the analyte for a given ligand level.
R_L, amount of ligand immobilized.
MW, molecular weight.
n, stoichiometry of the reaction.

Fig. 4.2. Determining theoretical R_{max}. The standard analyte response that gives the best kinetic analysis is between 100–200 RU. RU, response units.

Choosing a Sensor Chip for Amine Coupling

Which sensor chip is used to study the interaction will depend on the level of immobilization of ligand that is required and on the specific application. A complete guide to the different ProteOn sensor chips is given in Chapter 2. In short, for protein-protein interactions, GLC and GLM chips are sufficient. For protein–small molecule interactions, GLM and GLH chips are the best choice.

4.2.4 Deactivation

The injection of 1 M ethanolamine follows the ligand immobilization step and deactivates any unreacted sulfo-NHS ester groups. Default injection parameters are 30 µl/min for 5 min.

4.2.5 Stabilization

This step is generally performed to ensure that any noncovalently attached proteins that may still be electrostatically held at the sensor surface are removed prior to the analyte injection and interaction analysis.

Stabilization buffer is injected across the surface. The type of buffer ranges from running buffer to harsher solutions like 50 mM NaCl and 50 mM NaOH. Care should be taken not to use a stabilization buffer that is so harsh that the immobilized ligand is denatured. This will reduce its activity, affect the interaction analysis, and reduce analyte response. Use short injections of 30–60 sec with high flow rates of 100 µl/min to reduce this possibility. After the stabilization injection is complete, look for a stable baseline. If the baseline drifts, a second stabilization injection may be needed or a harsher buffer may be used.

Part 2: Capture of Ligand

For some applications, such as antibody screening or capturing proteins using tags, immobilization of the ligand of interest directly to the chip surface may not be desired. In such a case, selective capture of the ligand from a crude sample for subsequent analysis with an analyte may be preferred.

Major Steps for Ligand Capture

For a customized ligand capture chip surface:

1. Conditioning of sensor chip — conditioning is performed following the sensor chip initialization process using ProteOn regeneration solutions (refer to Chapter 5.2)
2. Immobilization of capture reagent or biomolecule (activation, immobilization, and deactivation) using a general-use sensor chips such as GLC, GLM, and GLH chips. If working with biotinylated molecules or histidine-tagged proteins, NLC, HTG, or HTE chips may be used and this step may be skipped.
3. Injection of solution containing ligand to be captured, such as crude supernatant or tissue culture lysate.
4. Removal of nonspecifically captured biomolecules and stabilization of ligand capture prior to interaction analysis.

4.2.6 Ligand Capture Using Capture Proteins — Antibody Screening

In some cases, capture of the ligand of interest from a crude sample, such as a hybridoma supernatant or phage display supernatant, prior to analysis with an analyte may be the method of choice. This type of noncovalent capture is ideally suited to the ProteOn XPR36 system, as the 6 x 6 array allows for the rapid screening of hundreds of antibodies.

Ligand Capture Conditions for mAb Screening

To capture a mAb from a crude hybridoma supernatant, create a sensor chip that contains a relevant capture protein to capture the mAb, such as an anti-IgG antibody or protein A/G. These anti-IgG and protein A/G surfaces can be created using the direct amine coupling method described previously.

Using R_{max} to Determine Ligand Capture Conditions for mAb Screening

Consideration must be taken to ensure that enough of the mAb is captured to be able to interact with its analyte. The level of mAb captured is dependent on the amount of mAb available in the supernatant and on the efficacy and immobilization level of the capture protein. In this case, the R_{max} equation must be used twice. First, determine how much mAb must be captured to be able to see an analyte response of ~200 RU. Second, calculate how much of the protein capture anti-IgG or protein A/G would need to be immobilized to attain the required mAb level.

4.2.7 Ligand Capture by Biotin Label or Histidine Tag — The NLC, HTG, and HTE Sensor Chip

The NLC chip allows for the selective capture of ligands that contain a biotin tag, such as proteins, DNA, or liposomes. The NLC chip comes prepared with NeutrAvidin immobilized to its surface. This chip is suitable for subsequent protein-protein and protein–nucleic acid interaction analysis.

The HTG and HTE chips feature a novel tris-NTA (3 x NTA) surface for stable capture of histidine-tagged proteins. The HTG chip has a compact capacity for protein-protein interaction analysis and the HTE chip has a high capacity for protein–small molecule interaction analysis. (Refer to Chapter 5, section 5.2 for the ligand capture conditions for NLC, HTG, and HTE chips.)

Table 4.1. Troubleshooting.

Problem	Possible Causes	Solution
Working with acidic proteins	Acidic proteins are difficult to immobilize by amine coupling, as they require buffer conditions that may be denaturing and may neutralize the activated negative sulfo groups on the chip surface and prevent attraction	Try a capture method or biotinylation of the acidic protein
Enhance immobilization	The amount of protein immobilized is too low	Increase contact time Lower flow rate Increase protein concentration Optimize pH
Ligand immobilized but no interaction	Protein may no longer be active because the immobilization conditions are too harsh (too strong pH or salts)	Use positive control to gauge the activity of the immobilized protein
	The active site on the protein may be buried because of the random immobilization orientation	Try a capture method to ensure correct orientation
	Enzymes may be active only if immobilized in the presence of another molecule or cofactor or to protect the active binding site	Immobilize in presence of protecting molecule or cofactor
Mass transport	This occurs when the rate of diffusion of the analyte from the flow is slower than the rate of association of the analyte to the ligand	Reduce ligand density or increase analyte flow rate

4.2.8 Summary

The protocols and methods provided in this chapter are meant as a foundation to create your own experimental protocols and methods specific to your individual research projects.

Exact optimal experimental conditions will vary according to the specific application. We strongly recommend the conditions be optimized and determined, as this will lead to consistent and high-quality SPR results.

4.3 Guide to Analyte Injection on the ProteOn XPR36 System

4.3.1 Introduction

A simple binding interaction analysis by SPR starts with the immobilization of ligand to the sensor chip surface, as described in section 4.2. This is followed by the addition of the analyte of interest to the buffer flowing over the ligand surface. The interaction of the ligand and analyte is measured by the SPR instrument as a change in refractive index over time. From this, the association (k_a or k_{on}), dissociation (k_d or k_{off}), and equilibrium (K_D) constants can be derived. These data are valuable to those studying biomolecular interactions in many applications from binding site interface analysis and concentration determination to thermodynamic analysis.

4.3.2 Full Kinetic Profile

To generate a full kinetic profile for the interaction of an analyte with a ligand and obtain the binding constants above, one must measure the interactions at multiple analyte concentrations. Typically, multiple analyte concentrations are required for good model fitting. The benefit to using the ProteOn XPR36 array system for kinetic analysis is that its 6 x 6 array lends itself perfectly to the simultaneous injection and analysis of up to six analyte concentrations at once. One of these analyte concentrations can be sacrificed for use as a real-time double reference.

4.3.3 Determination of Analyte Concentrations

As a rough guide, the range of concentrations needed for an analyte injection should span 10x greater than and 10x less than the expected K_D. If you are starting with an unknown system and you have no prior knowledge of the K_D, search the literature to discover if a similar interaction system has been previously studied to obtain guidance on where to start. If this is not possible, then consider what type of biomolecules you are working with. For example, if it is an antibody-antigen interaction, you would expect something within the nanomolar to subnanomolar K_D range for a tight interaction. If this is a completely novel system, then choose a very large range of concentrations for an initial scouting experiment. This allows you to home in on the concentration range, gradually decreasing the concentration range to span 10x above and below the K_D.

4.3.4 Analyte Preparation

Knowing your analyte concentration is key, as it directly affects k_a and k_d. Analyte samples should be created using serial dilution into running buffer to minimize bulk effects. Take care to avoid vortexing, as this will cause bubbles and destroy proteins. Samples may be centrifuged for about 15 seconds to ensure that all of the solution is at the bottom of the tubes prior to loading into the instrument.

4.3.5 Analyte Injection Parameters

Typically, analyte injections are performed at a high flow rate of about 50–100 µl/min; this helps to reduce any mass transport effects that may be present if a lower flow rate is used. Injections should be performed once the baseline is stable. A stabilization step may be needed in some cases. In order to calculate correct binding constants, the amount of time alloted to the association phase should be enough to observe a curvature of the binding response, and the dissociation phase should be long enough to observe decay in the response (Figure 4.3).

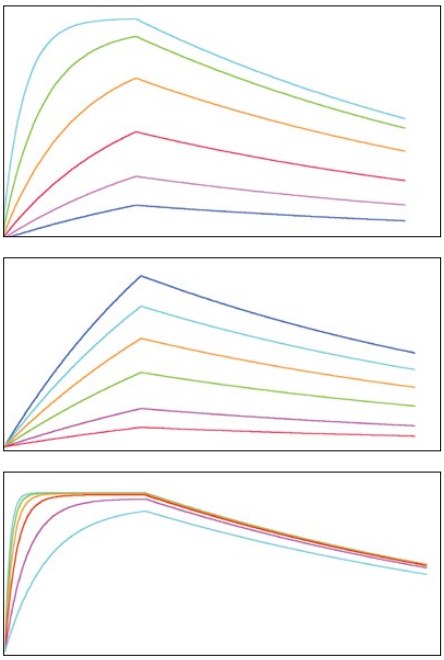


Fig. 4.3. Analyte concentrations and injection length. Extracting reliable binding kinetic constants requires: (**1**) the use of several analyte concentrations that bracket the K_D value and (**2**) injection length long enough to see a curvature of the binding response.

Initial analyte injection times can be guided by the strength of the interaction you are examining. If you are working with a small molecule that has fast binding and dissociation from the ligand surface, then you can keep the times short: between 1–2 min association and 1–2 min dissociation. See the table below for guidance on analyte injection times.

Table 4.2. Analyte injection time.

Type of interaction	Association, min	Dissociation, min
Fast binding/fast dissociation	1–2	1–10
Slow binding/slow dissociation	5	10–60
Fast binding/slow dissociation	1–2	10–60

4.3.6 Analysis of Binding Results

Once you have optimized the analyte injection parameters and concentrations to generate good quality reproducible data, you are ready to perform data processing and analysis. This analysis allows you to determine the type of interaction you are dealing with and to obtain binding kinetics, concentration, or thermodynamic parameters. Please refer to sections 4.4 and 4.5.

4.4 Guide to SPR Data Processing on the ProteOn XPR36 System

4.4.1 Interaction Sensorgram Terms

An SPR sensorgram is a graph of time-traced SPR responses during a biomolecular interaction analysis. The x-axis is time in seconds and the y-axis is SPR response in response units (RU). It should be noted that both ligand immobilization and analyte injection steps generate sensorgrams, but the sensorgram in the analyte injection step is more often used because it contains the ligand-analyte interaction information for affinity or kinetic analysis. Therefore, where not otherwise specified, "sensorgram" hereinafter refers to that generated in the analyte injection step.

As illustrated in Figure 4.4, an SPR sensorgram can be divided into three different phases:

Baseline is the phase before the injection of the analyte. Running buffer flows over the sensor chip surface bearing the immobilized ligand and the baseline response is recorded.

Association is the phase during the injection of the analyte. The analyte flows over the sensor chip surface and binding occurs between the ligand and the analyte. A concave increasing response curve is produced. Depending on the binding kinetics of the interaction partners and the experimental conditions, the increasing response curve may or may not reach a plateau, which indicates that interaction equilibrium has been reached.

Dissociation is the phase after the injection of the analyte is finished. Running buffer flows over the sensor chip surface and washes off the analyte bound to the ligand. A convex decreasing response curve is produced. Depending on the binding kinetics of the interaction partners and the experimental conditions, the decreasing response curve may or may not return to baseline, which indicates complete dissociation of the analyte from the ligand surface.

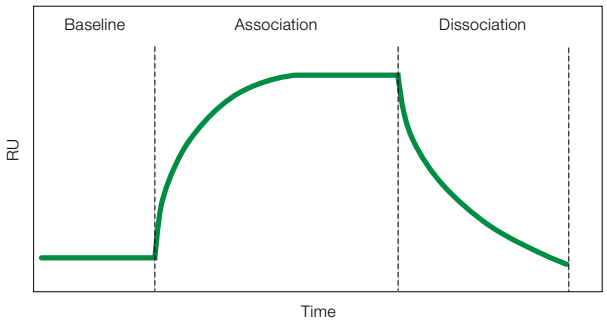


Fig. 4.4. A typical SPR sensorgram displaying the three sensorgram phases. RU, response units.

Kinetic analysis requires the presence of all three phases, whereas equilibrium and concentration analyses do not. However, note that (1) the association phase must reach the interaction equilibrium plateau for an equilibrium analysis, and (2) the beginning of the association phase must be linear to reveal mass-transport limitations to kinetics in a concentration analysis.

4.4.2 Sensorgram Display

In the 6 × 6 experimental configuration of the ProteOn XPR36 system, 36 sensorgrams are produced simultaneously in each SPR experiment. In the sensorgram display, by default the sensorgrams are grouped by ligand in six sensorgram windows, or graphs. In the One-shot Kinetics™ approach, each graph contains a set of six dose-response sensorgrams for a dilution series of the analyte interacting with six identical ligand surfaces. During data processing, the grouping of sensorgrams can be adjusted in the **Data Grouping** screen, and individual sensorgrams can be removed from or added back to the sensorgram display in the **Interaction** screen.

The sensorgram graphs can be selected or deselected by clicking their blue title bars. In the menu bar, click **View** and **Select All Graphs** to select all the sensorgram graphs. While **Auto** data processing activities apply to all the sensorgram graphs, **Selected** data processing activities apply only to the selected sensorgram graphs.

4.4.3 Sensorgram Processing

Align the Sensorgram Set

To perform reliable kinetic analysis, a set of dose-response sensorgrams for the same interaction are typically analyzed together to minimize system deviations. This action requires the alignment of the graphs in a sensorgram set in both the vertical and horizontal dimensions. In scientific experiments, it is common to perform diagram or sensorgram alignment when processing multiple data groups in a dataset. The alignment action for SPR sensorgrams is similar to those performed for other bioanalytical technologies such as spectroscopy. ProteOn Manager™ software offers automatic injection alignment along the x-axis and baseline alignment along the y-axis available in the **Process** menu, as shown in Figure 4.5.

Injection alignment adjusts all the sensorgrams to share the same starting point along the x-axis, thus removing time differences among the different sensorgrams. Unlike traditional serial-flow SPR systems, the ProteOn XPR36 system has a parallel flow design that allows for synchronized analyte injection across different flow channels. Therefore, injection alignment is typically achieved with very good accuracy.

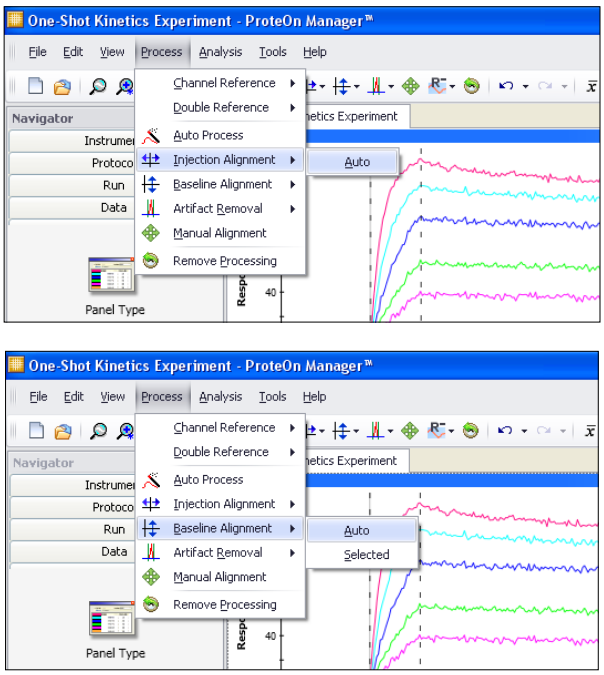


Fig. 4.5. The automatic injection alignment and baseline alignment functions offered in ProteOn Manager software.

Baseline alignment adjusts all the sensorgrams to the same zero-baseline level along the y-axis, thus removing slight baseline-level differences among the sensorgrams resulting from previous steps. Although the automatic baseline alignment function processes entire sensorgrams by default, it is possible to align the sensorgrams based on the values in a selected region. To define the selected region, click and drag in the sensorgram graph. Click **Process**, select **Baseline Alignment**, and choose **Selected** in the submenu to perform baseline alignment for the selected region.

In addition to the alignment options described above, manual correction may be applied to fine-tune the sensorgrams. To perform the manual sensorgram alignment, click **Process** and select **Manual Alignment**. Individual sensorgrams can then be moved when selected with the mouse.

Remove the Artifacts

Sensorgram artifacts, usually spikes caused by tiny air bubbles in the analyte solution, are sometimes present and should be removed. Note that *artifact* here refers to a response deviation over a very small time period. If a significant portion of the sensorgram deviates from the expected response, the trial should be rerun.

ProteOn Manager software offers automatic artifact removal that flattens these artifacts to restore sensorgram integrity, as illustrated in Figure 4.6. Although the automatic artifact removal processes entire sensorgrams by default, it is possible to process a selected region of the sensorgrams. To define the selected region, click and drag in the sensorgram graph. Click **Process**, select **Artifact Removal**, and choose **Selected** in the submenu to perform artifact removal within the selected region.

For ease of use, ProteOn Manager software allows the user to conduct all the sensorgram processing steps with a single command. Selecting **Auto Process** in the **Process** menu sequentially performs injection alignment, baseline alignment, and artifact removal.

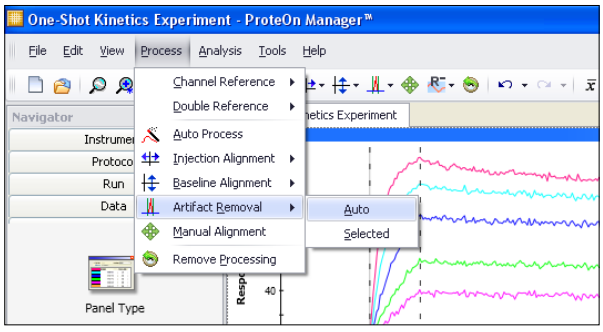


Fig. 4.6. The automatic artifact removal function offered in ProteOn Manager software.

4.4.4 Sensorgram Referencing

Sensorgram referencing is the most important step in data processing. The subtraction of references removes artifacts of refractive index change (bulk effect) from the analyte sample, nonspecific binding (NSB) of the analyte and impurities on the sensor chip surface, and changes of the ligand surface. There are two types of referencing in SPR analysis: blank surface referencing and blank buffer referencing. A blank surface reference is used to correct for bulk effect and NSB, and a blank buffer reference is used to correct for baseline drift resulting from the changes of the ligand surface.

The novel 6 × 6 experimental configuration in the ProteOn XPR36 system offers a comprehensive set of referencing options. The referencing options can be selected from the **Process** menu, as shown in Figure 4.7. The available referencing options are listed below.

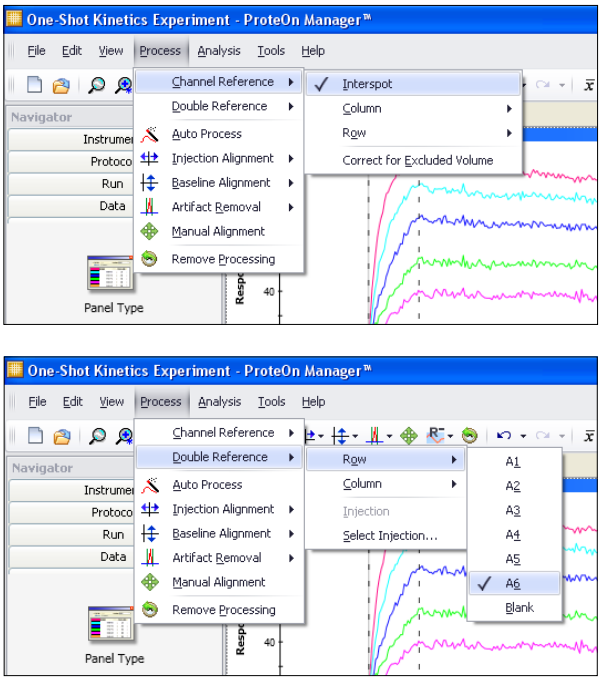


Fig. 4.7. ProteOn Manager software presents all the referencing options available in the ProteOn XPR36 system for selection.

Blank surface referencing (also known as channel referencing) is performed on a blank surface (either an empty or an irrelevant protein-coated surface) with an analyte solution flowing over it. The reference responses are collected on blank surfaces during the analyte injection (that is, blank surface reference = blank surface + analyte solution), as shown in Figure 4.8. Blank surface referencing is used to correct for bulk effect and NSB.

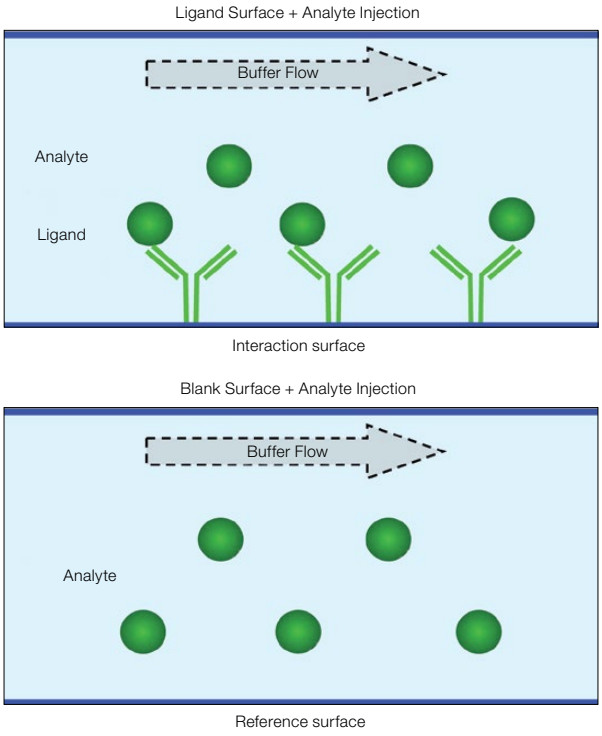


Fig. 4.8. Schematic diagram of a blank surface reference.

The ProteOn XPR36 system offers two blank surface referencing options, as illustrated in Figure 4.9.

Channel referencing is the reference method traditionally used in commercial SPR biosensors. It involves reserving a portion of the potential interaction surfaces for use as blank surfaces.

Interspot referencing is unique to the ProteOn XPR36 system. Instead of consuming potential interaction surfaces, this reference method employs the interval surfaces adjacent to interaction surfaces. Compared with the traditional channel reference, the interspot reference has the advantages of immediate proximity to interaction spots and the conservation of interaction spots. The immediate proximity enhances the referencing quality.

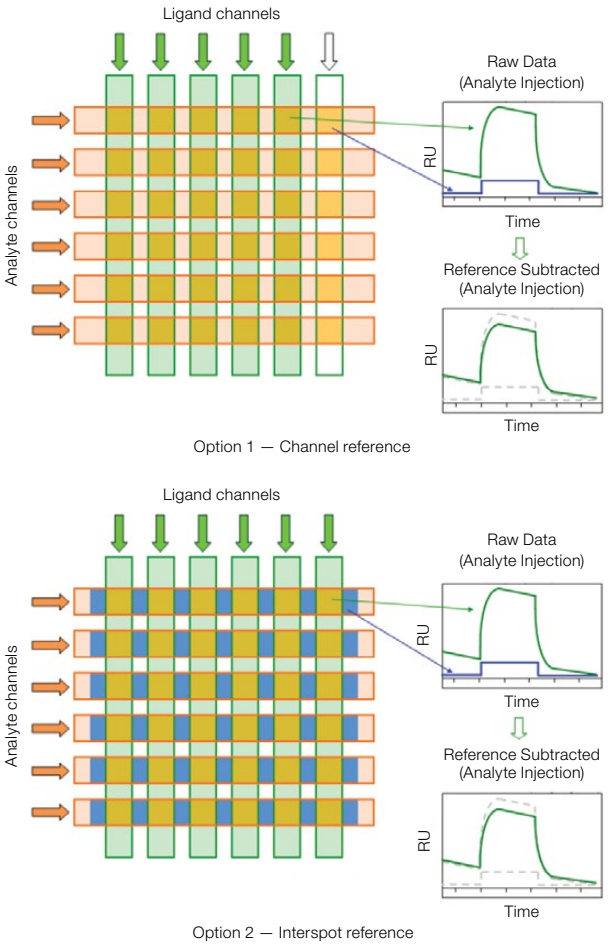


Fig. 4.9. The two blank surface referencing options in the ProteOn XPR36 system. RU, response units.

Blank buffer referencing (also known as double referencing) is performed on a ligand surface with a blank buffer (either a running buffer or a negative control sample) flowing over it. The reference responses are collected on ligand surfaces during a blank buffer injection (that is, blank buffer reference = ligand surface + blank buffer), as shown in Figure 4.10. Blank buffer referencing is used to correct for baseline drift resulting from the changes of the ligand surface.

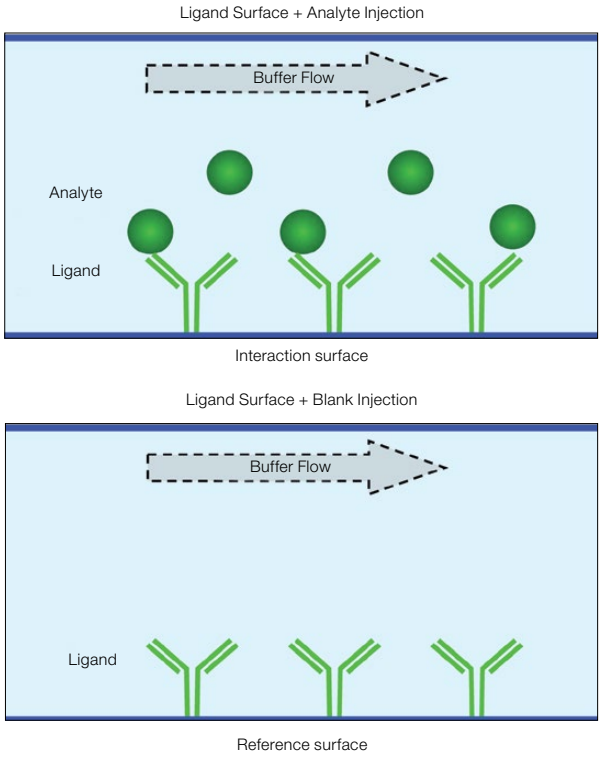


Fig. 4.10. Schematic diagram of a blank buffer reference.

The ProteOn XPR36 system offers two blank buffer referencing options, as illustrated in Figure 4.11.

Injection referencing is the reference method traditionally used in commercial SPR biosensors. It requires a blank buffer injection performed prior to the analyte injection.

Real-time double referencing is unique to the ProteOn XPR36 system. This method employs a blank buffer injection in parallel with the analyte injection. Compared with the traditional injection reference, the real-time double reference has the advantages of accurate monitoring of possible changes on ligand surfaces and saving time by eliminating the additional blank buffer injection. The accurate monitoring of ligand surfaces greatly enhances the referencing quality, especially in the cases of capture surfaces, where reversible capture of the ligand is employed and exponential baseline decay is often observed.

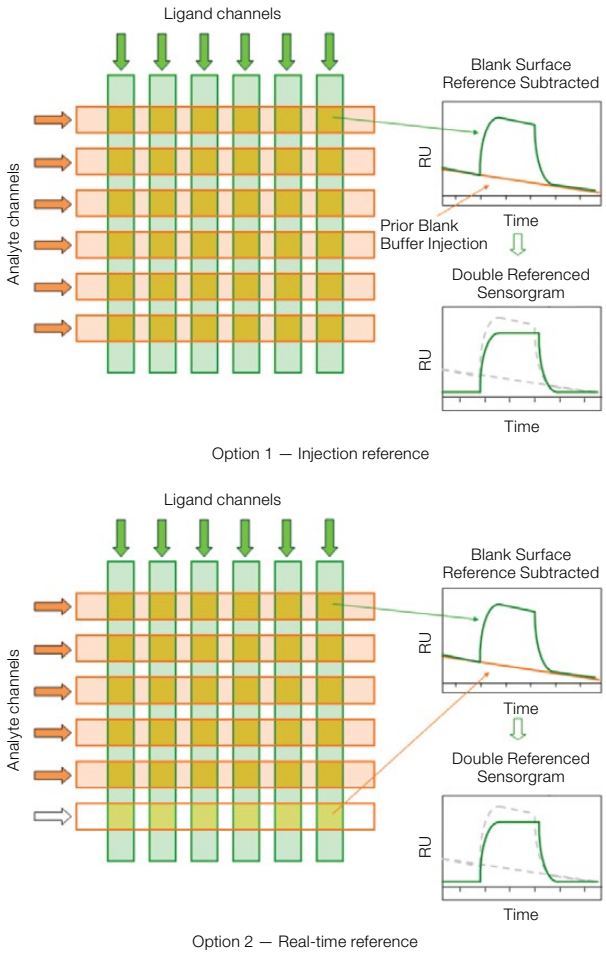


Fig. 4.11. The two blank buffer referencing options in the ProteOn XPR36 system. RU, response units.

Referencing options should be selected in the experiment design phase, as the reference surfaces are created in the ligand immobilization and analyte injection steps. A combination of blank surface and blank buffer referencing is usually applied to yield high-quality SPR results. This combination is implemented by sequentially subtracting one blank surface reference and one blank buffer reference. The ProteOn XPR36 system offers the flexibility of selecting any combination of the four references to optimize data processing.

Excluded volume correction (EVC) is not an independent referencing option but rather a calibration with a blank surface reference. This calibration is applied when a cosolvent with a high refractive index, such as DMSO, is used in an analyte solution to increase the analyte solubility. A high refractive index cosolvent may produce a larger bulk effect on a reference surface than on an interaction surface due to the volume exclusion of the cosolvent by the ligand on the interaction surface. This inconsistency can be resolved by the EVC calibration. Please refer to section 4.6 for a detailed explanation of this calibration and experimental guidance.

4.4.5 Quality Standards for Processed Sensorgrams

The following standards are used to judge the quality of processed sensorgrams:

1. **Processed sensorgrams** — the sensorgrams are aligned in both dimensions, and artifacts, such as air bubbles, are removed. This processing requires both good-quality raw sensorgrams and appropriate software functions.
2. **Good choice of referencing** — both blank surface referencing (channel referencing) and blank buffer referencing (double referencing) are appropriately performed. The experimental design must ensure the incorporation of the correct referencing options. The referenced sensorgrams should not show bulk effects or baseline drift. Although it is not required, the best practice is to have no response jump present between the end of the association and the beginning of the dissociation phases.
3. **Sufficient interaction time** — the interaction time or the time of analyte injection in the association phase is long enough to show curvature, and the running buffer injection time in the dissociation phase is long enough to show adequate response decrease to resolve the dissociation rate constant. The choice of appropriate injection conditions, including interaction time, analyte concentration, and injection flow rate, is based on the user's understanding of the interaction. For example, the user can determine the binding affinity and ligand-analyte complex stability by obtaining this information from either preliminary experimental trials or literature values. This consideration is essential for accurate sensorgram fitting.

Note: The steps of data processing and data analysis using ProteOn Manager software are outlined in section 4.7.

4.5 Guide to SPR Data Analysis on the ProteOn XPR36 System

4.5.1 Kinetic Analysis

To determine the kinetic constants of a biomolecular interaction through SPR analysis, the sensorgram must be fitted to a kinetic model using a mathematical algorithm. In ProteOn Manager software, the user may choose among seven different binding models with which to perform the interaction analysis. However, it is recommended that SPR interactions are fitted to the simplest model possible.

Binding Models — Langmuir

The most commonly used binding model for SPR biosensors is the Langmuir model. It describes a 1:1 interaction in which one ligand molecule interacts with one analyte molecule. In theory, the formation of the ligand-analyte complex follows second-order kinetics. However, because the majority of SPR biosensors are fluidics-based and capable of maintaining a constant analyte concentration in a continuous liquid flow, complex formation actually follows pseudo-first-order kinetics. In addition, this model assumes that the binding reactions are equivalent and independent at all binding sites. It is also assumed that the reaction rate is not limited by mass transport. Many interactions adhere to this model, in which the interaction is described by the simple equation shown below, where B represents the ligand, and A is the analyte. The rate of complex formation is represented by the association constant (k_a, in the unit of $M^{-1}s^{-1}$) and the rate of complex decay is represented by the dissociation constant (k_d, in the unit of s^{-1}), as given by Equation 1:

Association Phase

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

Equation 1

In a kinetic analysis, the equilibrium constant (K_D, in the unit of M) is calculated from the two kinetic constants through the defining relation $K_D = k_d/k_a$. Relating the interaction state to the SPR sensorgram is accomplished by applying specific equations relevant to the different sensorgram phases, as illustrated in Figure 4.12.

Association: $$R_t = \frac{R_{max}\,[A]}{K_D + [A]}\left[1 - e^{-(k_a[A] + k_d)t}\right]$$

Equation 2

Dissociation: $$R_t = R_0 e^{-k_d t}$$

Equation 3

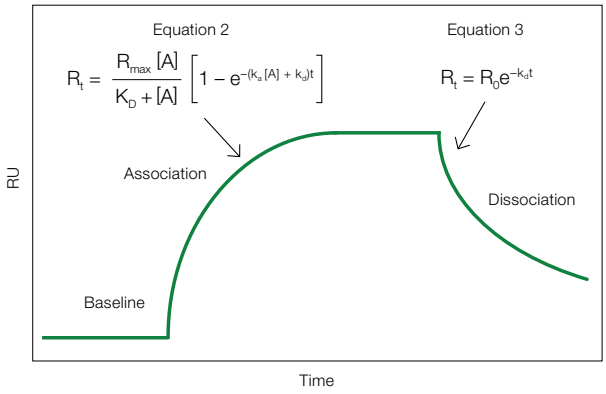


Fig. 4.12. An idealized sensorgram showing the baseline, association, and dissociation phases.

Analysis of the sensorgram curve in the association phase, in which binding is measured while the analyte solution flows over the ligand surface, allows the determination of the rate of complex formation. There is an associated increase in response units over time as the complex forms on the chip surface. Figure 4.13 outlines the derivation of Equation 2.

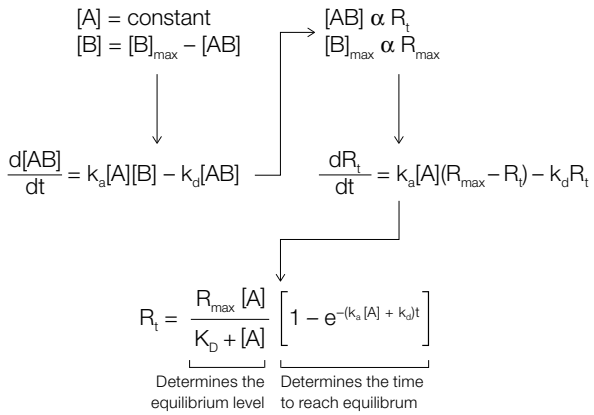


Fig. 4.13. The derivation of Equation 2.

As can be inferred from this derivation, the change in the amount of complex formed over time is linearly related to k_a, k_d, and the analyte concentration, [A]. The complex formation can be further described in terms of response units, where the change in response units over time is again linearly related to k_a, k_d, and [A]. Thus, Equation 2 describes the level of response at equilibrium and also the time taken to reach a certain response level during the association phase.

Dissociation Phase

In the dissociation phase, the analyte concentration in the flow is suddenly reduced to zero by the injection of running buffer. The rate of complex dissociation follows simple exponential decay, or first-order kinetics. Equation 3 is derived in a manner similar to Equation 2. It describes the time taken to reach a certain response level during the dissociation phase, as outlined in Figure 4.14.

$$d[AB]/dt = k_a[A][B] - k_d[AB] \longleftarrow [A] = 0$$

$$\downarrow$$

$$d[AB]/dt = -k_d[AB]$$

$$\downarrow$$

$$dR_t/dt = -k_dR_t$$

$$\downarrow$$

$$R_t = R_0e^{-k_dt}$$

R_0 is the signal level at the beginning of dissociation.

Fig. 4.14. The derivation of Equation 3.

ProteOn Manager software offers two options for the Langmuir model: simultaneous k_a/k_d or off-rate analysis. The first option is the default choice, which fits both the association and dissociation phases for the full set of constants (k_a, k_d, and K_D), whereas the second option analyzes only the dissociation phase for k_d.

Langmuir with Drift or Mass Transport

There are two other kinetic interaction models based on the Langmuir equations: Langmuir with drift and Langmuir with mass transport. Langmuir with drift is commonly used in experiments that use a capture surface, for example, the reversible antibody or histidine-tag capture surface. In such cases the captured ligand may escape from the capture reagent on the chip surface, leading to baseline drift before the analyte injection and during the association and dissociation phases. Note that this model calculates only a linear drift that is constant with time. Blank buffer referencing should be used to gain accuracy when correcting for a large baseline drift showing an exponential curvature as described in section 4.4.

The second model is Langmuir with mass transport. Mass transport is the process whereby an analyte diffuses from the bulk solution to the chip surface. To determine whether a particular interaction is limited by mass transport and thus whether this model should be used, inject an analyte sample at different flow rates. If the association curves are different, then this interaction is mass-transport limited. In contrast, if the association curves are independent of the flow rate (all binding curves overlap), then diffusion is not the rate-limiting factor, and the simple Langmuir model can be applied.

Other Binding Models

There are four complex binding models for analyzing non-Langmuir interactions: the heterogeneous analyte, heterogeneous ligand, two-state, and bivalent analyte models. When choosing a model to determine the binding kinetics of interactions, the Langmuir or Langmuir with mass transport model should be selected by default since the majority of biological interactions occur in a 1:1 stoichiometry. It is necessary to provide a biological justification for the use of other models, and conclusions based on analyses with these complex models should be confirmed with additional experiments.

Langmuir Model with Drift

The Langmuir model with drift is used when a biomolecular interaction follows simple 1:1 binding but exhibits a persistent baseline drift that interferes with data interpretation. This is applied in SPR experiments using capture agents, as the captured ligand may leach from the surface over time. The Langmuir model with drift uses the same kinetic equations as the simple Langmiur model but calculates the drift as a linear drift with time, D*t, where D is the slope of the drift. It should be noted that this model should be applied to the experiments with slow baseline drift because fast baseline drift caused by the rapid decay of the captured ligand usually shows an exponential curvature and does not fit with this model. The optimal solution is correcting the baseline by the subtraction of a blank buffer reference (reference of blank analyte buffer over ligand surface).

Langmuir Model with Mass Transport Limitations

The Langmuir model with mass transport limitations assumes a 1:1 binding model, as is the case with the simple Langmuir model, but it takes into account the rate at which analyte is brought from the bulk solution to the sensor chip surface, which is governed by mass transport. Some biomolecular interactions may be mass transport limited if the rate of association is faster than the rate at which analyte diffuses to the sensor chip surface. The following equation describes Langmuir binding with mass transport limitations:

$$A_{bulk} \underset{k_t}{\overset{k_t}{\rightleftharpoons}} A_{surface} + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

Equation 4

where k_t is the mass transport rate constant for the diffusion of analyte A from the bulk solution to the surface. A good test of whether an interaction is mass transport limited is to run the experiment at different flow rates and calculate the association rate constant. Diffusion to the surface of the sensor chip will be faster at higher flow rates; thus, if the association rate of a given interaction increases with higher flow rates and decreases with low flow rates, most likely the interaction is mass transport limited. Usually one can get around a mass transport limited interaction by running the ProteOn XPR36 system at high flow rates or by using low ligand density; however, there are certain situations when even these adjustments cannot eliminate the mass transport effect, and modeling the interaction using a Langmuir model with mass transport limitations is more attractive.

Bivalent Analyte Model

The bivalent analyte model is used when an analyte has two separate binding sites. The following equation describes binding of a bivalent analyte:

$$A + B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} AB + B \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} ABB$$

Equation 5

where A is the analyte and B is the ligand. The association and dissociation of the first binding event is described by k_{a1} and k_{d1}, respectively, while k_{a2} and k_{d2}, respectively, describe the association and dissociation of the second binding event. The first event will yield a traditional 1:1 kinetic fit where the second binding event will cause the ligand-analyte complex to stabilize, thus changing the kinetics of the reaction. Therefore, a sensorgram of a bivalent analyte binding to ligand is the result of two separate kinetic processes occurring in tandem.

Heterogeneous Analyte Model

When an analyte is heterogeneous, analyte may bind to the ligand in two different locations. This can occur naturally if a sample is not completely pure or if there are two different types of analyte in solution. Thus, a sensorgram of a heterogeneous analyte binding to immobilized ligand represents the sum of two separate binding interactions. If one analyte has a naturally higher affinity than the other analyte, the two may compete for binding of the ligand and the sensorgram data will reflect the binding kinetics of the higher affinity ligand. The following equations are used to describe and model the binding of a heterogeneous analyte:

$$A1 + B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} A1B;\ A2 + B \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} A2B$$

Equation 6

Heterogeneous Ligand Model

A heterogeneous ligand model assumes that there are two sites on the ligand that bind analyte. This can occur if ligand binds to the sensor chip in different orientations, resulting in different binding faces being presented to the analyte. Polyclonal antibodies recognize different epitopes on the same antigen and thus would be considered a heterogeneous ligand. The following equation describes binding of analyte to a heterogeneous ligand:

$$A + B1 \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} AB1;\ A + B2 \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} AB2$$

Equation 7

where B1 and B2 are the two separate binding sites on the ligand and A is the analyte. Note that there are two separate sets of association and dissociation rate constants (k_{a1}/k_{d1} and k_{a2}/k_{d2}) to describe each binding event. The binding response of a sensorgram from a heterogeneous ligand then, is the sum of the binding response of two separate binding events.

Two-State Conformation Model

The two-state conformation model accounts for the existence of two conformations of the bound complex. This can happen if binding of the analyte to ligand triggers a change in conformation of the bound complex. Equation 8 describes the two-state confirmation binding model:

$$A + B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} AB \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} (AB)^*$$

Equation 8

In Equation 8, AB is the first conformation of the bound complex and (AB)* is the second conformation of the bound complex. Once the complex AB forms it can either dissociate to unbound ligand (B) and free analyte (A) or change to the new conformation (AB)*. However, the complex (AB)* must return to the first complex AB before dissociating into unbound ligand and free analyte. The two-state conformation model is very useful for describing an allosteric binding effect where binding of analyte to ligand (a substrate or inhibitor binding to an enzyme, for example) results in a conformational change.

Parameter Setting

In ProteOn Manager software, there are two parameter setting options for kinetic analysis: (1) the choice of **Fitted** or **Constant** parameters, and (2) the choice of **Global**, **Grouped**, or **Local** sensorgram fitting scopes, as indicated in Figure 4.15.

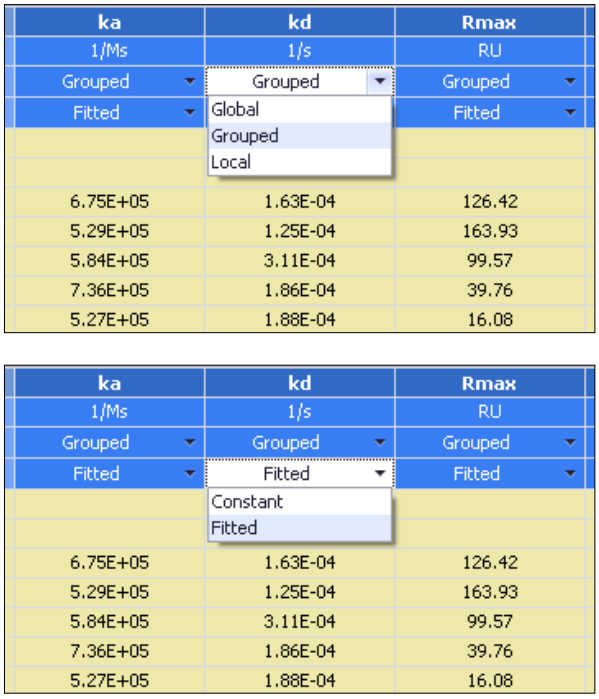


Fig. 4.15. The parameter setting options for kinetic analysis.

The fitted parameters are variables in the sensorgram fitting, whereas constant parameters are fixed at their initial values. The three sensorgram fitting scopes — global, grouped, and local — are defined and compared in Table 4.3. When setting the parameter fitting types, the initial values of all parameters can be changed to start the sensorgram fitting from a closer point to the result. This option may be applied in sensorgram fitting with complex models to reduce computational demands.

Table 4.3 shows the results of an experiment with the ProteOn protein–small molecule kit. In this experiment, six identical ligand channels were prepared so that the global fitting of all 36 sensorgrams and the grouped fitting of a set of six sensorgrams in each ligand channel are comparable.

Table 4.3. Results of an experiment using the ProteOn protein-small molecule kit.

Parameter	k_a (1/Ms)	k_d (1/s)	K_D (M)	R_{max} (RU)	Chi^2 (RU)
Scope	Global	Global	Global	Global	All
	1.51×10^4	3.63×10^{-2}	2.41×10^{-6}	80.52	6.0
Scope	Grouped	Grouped	Grouped	Grouped	All
L1	1.58×10^4	3.76×10^{-2}	2.38×10^{-6}	84.9	5.3
L2	1.60×10^4	3.75×10^{-2}	2.35×10^{-6}	78.7	5.0
L3	1.54×10^4	3.67×10^{-2}	2.38×10^{-6}	79.3	5.1
L4	1.54×10^4	3.54×10^{-2}	2.30×10^{-6}	78.6	5.8
L5	1.42×10^4	3.56×10^{-2}	2.51×10^{-6}	80.4	5.8
L6	1.36×10^4	3.48×10^{-2}	2.56×10^{-6}	81.3	8.1

Global: Parameters are identical for all sensorgrams.
Grouped: Parameters are identical for a certain ligand channel.

4.5.2 Equilibrium Analysis

The equilibrium constant, K_D, can be calculated directly from a sensorgram using Equation 9:

$$R_{eq} = \frac{R_{max}[A]}{K_D + [A]}$$

Equation 9

Equation 9 describes the response at the steady-state or equilibrium phase of the interaction, as shown in Figure 4.16. In this phase, the rate of association equals the rate of dissociation. To determine the K_D, the response at equilibrium, R_{eq}, is measured over a given range of analyte concentrations and the values are plotted as shown in Figure 4.17. R_{eq} is proportional to the analyte concentration at the low concentration range, but as the analyte concentration is increased, it approaches the theoretical maximum response R_{max},

the limiting value. When performing an equilibrium analysis, use data in which the responses of all analyte concentrations have reached equilibrium and confine the fitted region to the areas where the responses are flat.

Note that in ProteOn Manager software, the equilibrium analysis also presents the choices of fitted or constant and global or grouped for parameter setting. The definitions are the same as those described in the previous section.

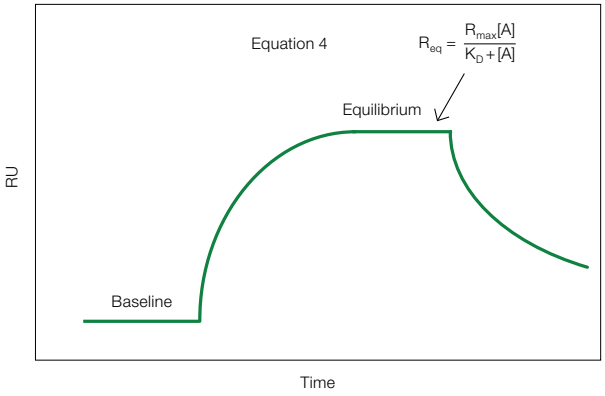


Fig. 4.16. An idealized sensorgram displaying the equilibrium phase.

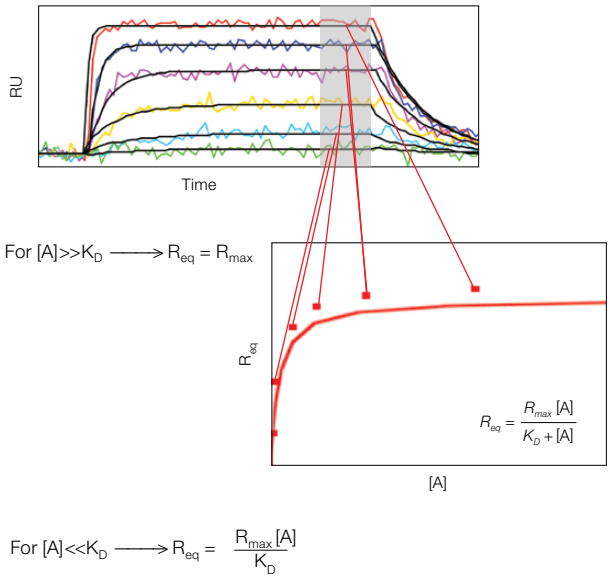


Fig. 4.17. Determination of the equilibrium constant.

4.5.3 Concentration Analysis

Although SPR biosensors can be used to determine analyte concentrations at binding equilibrium in a manner similar to an enzyme-linked immunosorbent assay (ELISA), concentration analysis in SPR biosensors is usually implemented in a different approach for higher efficiency and convenience. Here, the initial binding rate of a biomolecular interaction is measured under mass transport limited conditions, in which the binding rate is directly proportional to the bulk analyte concentration, as shown in Equation 10. The concentration of an unknown sample is calculated by comparing the binding response under these conditions to a standard curve of binding responses for known concentrations, as shown in Figure 4.18.

$$\text{Initial Binding Rate} = k_t [A] \qquad \textbf{Equation 10}$$

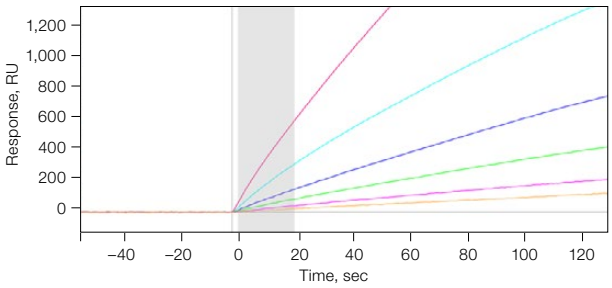


Fig. 4.18. The mechanism of concentration analysis. k_t is the mass transport coefficient in the unit of RU $M^{-1}s^{-1}$.

In ProteOn Manager software, the standard four-parameter logistic equation is employed to determine the unknown concentration. Note that the concentration analysis again presents the scopes of fitted or constant and global or grouped for parameter setting. The definitions are the same as those described in the previous section.

4.5.4 Report Point

A report point is created to directly read the average value of sensorgrams within a specified time range. Sensorgram fitting is not involved in this procedure. A report point is often used to measure the immobilization level of ligands or qualitatively compare the responses with different analyte injections. A report point is created in two steps: (1) right click and drag to select a time range, and (2) right click the selected time range to create a report point. The report point values are shown in a new column of the data table, as shown in Figure 4.19. The report point created in another dataset may be imported to the current dataset by right clicking the data table and choosing **Add Report Point**.

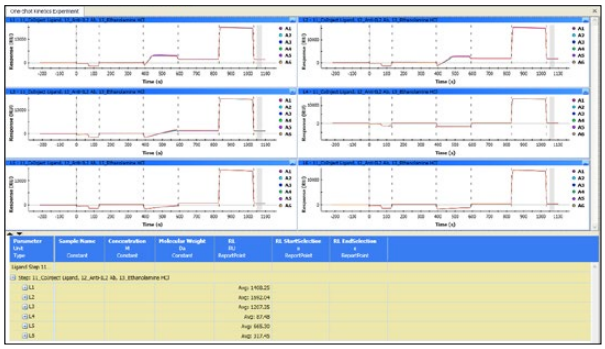


Fig. 4.19. Report point values are shown in a new column of the data table.

4.5.5 Data Presentation

ProteOn Manager software presents the results of an interaction analysis as a data table. The data table shows all the fitted parameters by default. The parameter list can be changed by dragging the parameter columns in or out of a parameter database **Column Chooser** to customize the parameter list. This option is available in the pop-up menu by right clicking the data table.

The data table is automatically grouped in the same way as the sensorgram sets (typically by ligand channels) and may be ungrouped to display all the values, as shown in Figure 4.20. In addition, the data table panel can be resized by clicking the arrow buttons at the top-left corner. The values in the data table can be selected and copied to spreadsheets.

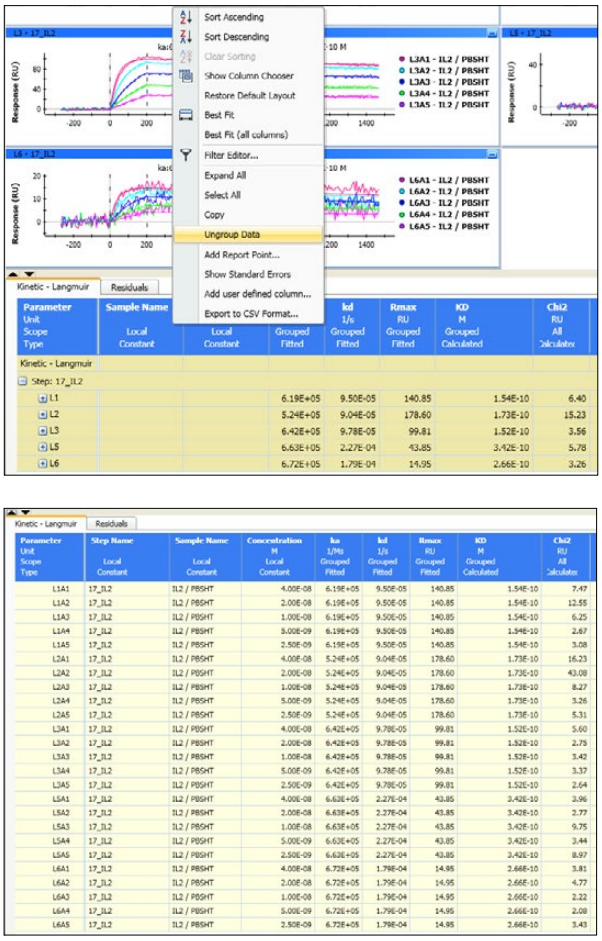


Fig. 4.20. The grouping and ungrouping of the data table.

In drug discovery research, isoaffinity and screening graphs are frequently used to view the screening results for positive-hit pickup. ProteOn Manager software can be used to create either graph. In the **Analysis** menu, choose **Isoaffinity Graph** or **Screening Graph** to display the graph. Both graphs allow the selection of target datasets in the left panel. Examples of an isoaffinity graph and a screening graph are presented in Figure 4.21.

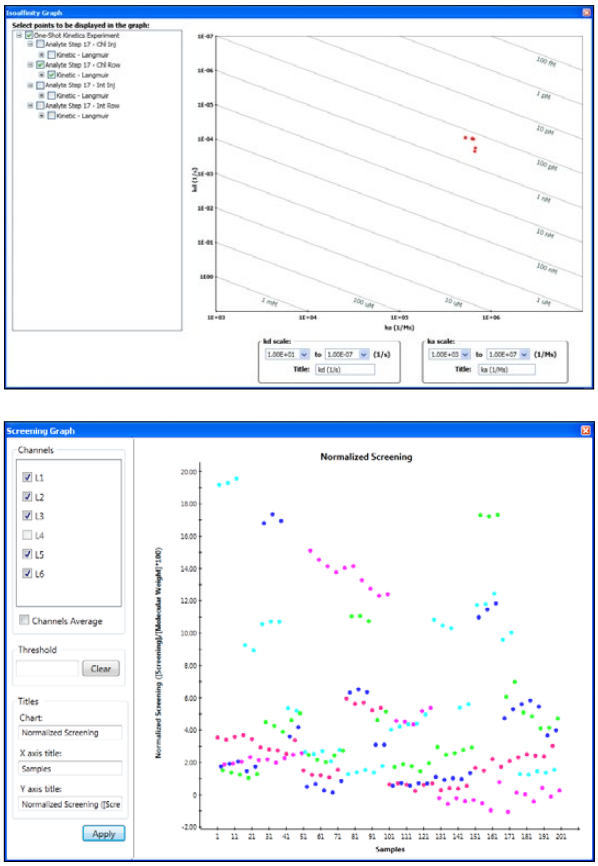
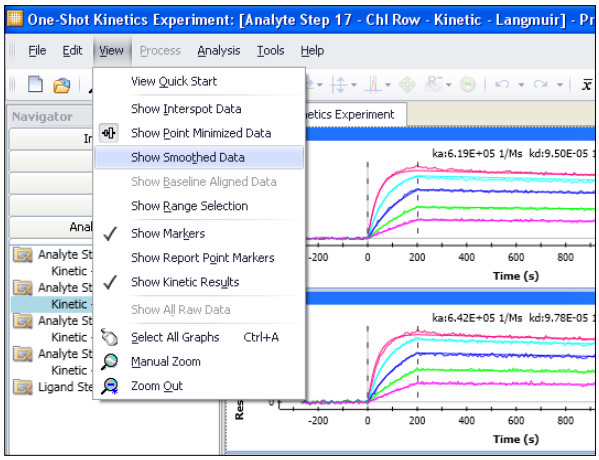


Fig. 4.21. Examples of an isoaffinity graph (top) and a screening graph (bottom).



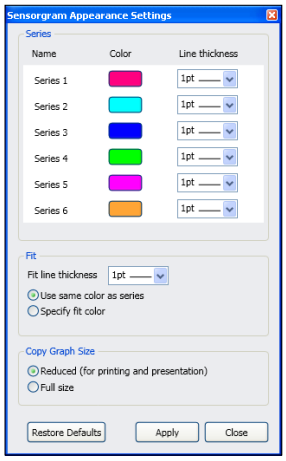


Fig. 4.22. The sensorgram smoothing and sensorgram appearance setting functions in ProteOn Manager software.

Note: Please refer to section 4.8 for the different options of exporting SPR results to external software platforms from ProteOn Manager software.

4.5.6 Sensorgram Appearance

To customize the sensorgram visualization to meet user preferences, ProteOn Manager software offers two appearance functions: sensorgram smoothing and sensorgram appearance setting.

Sensorgram smoothing is available in the **View** menu and may be removed by deselecting it. This function smoothes the baseline noise to better display the curvature of sensorgrams. Note that the sensorgram smoothing is only a display-related feature. It does not affect sensorgram fitting or SPR results because the data analysis is always based on the raw data.

The sensorgram appearance setting is available in the **Tools** menu and allows the user to choose sensorgram color and line thickness. The appearance change will be applied to the sensorgram series (a particular sensorgram in all sensorgram sets) rather than a single sensorgram. To change the color of a single sensorgram, open the **Interaction** screen in the **Data** tab, and right click an interaction spot to select a color. The sensorgram appearance functions in ProteOn Manager software are shown in Figure 4.22.

4.5.7 Quality Standards for SPR Results

The following standards are used to judge the quality of SPR results. An example of high-quality SPR results is shown in Figure 4.23, as a reference for applying the standards.

Visual inspection — the lines of the resulting fit should pass through the experimental sensorgrams. Both the fitted and original data should be displayed for publication

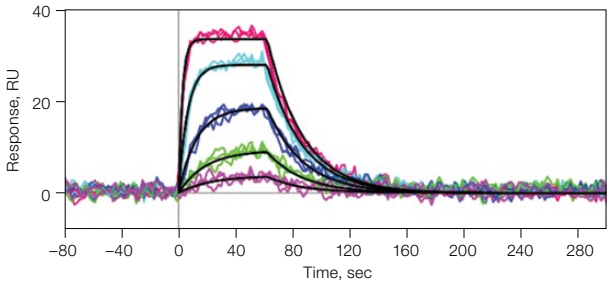
Parameter results — fitted parameters should be within an expected and reasonable range. The R_{max} value should be within the range of a few hundred RU, ideally less than 200 RU, to minimize the impact of mass transport effects. ProteOn Manager software provides the choices of global, grouped, or local sensorgram fitting. When available, it is recommended to compare the results of global and grouped analyses, to demonstrate the reliability of the sensorgram fitting. If it is possible to perform both kinetic and equilibrium analyses on the same dataset, the calculated K_D value obtained from the equilibrium analysis should be similar to the K_D value calculated from the individual k_a and k_d values obtained from the corresponding kinetic analysis. These two comparisons are usually applied to determine the confidence level of the fitted parameters. The fitted parameters must be recorded when publishing SPR results

Chi² — Chi^2 is the average of the squared residuals (the average of the squared differences between the measured data points and the corresponding fitted values). The lowest value that can be expected is the baseline noise. The Chi^2 value should also be published, as it indicates the fitting confidence. Empirically, these values should be less than 10% of R_{max} regardless of units

Residuals — a plot of the residuals should form a random scattering of the same order of magnitude as the noise level. It is helpful to display the residual data along with the fitted data when publishing your work

Standard errors — standard errors determine how sensitive the sensorgram fitting is to changes in the parameters and should be included in publications

Signal-to-noise ratio — the responses in both the association and dissociation phases must show an adequate signal-to-noise ratio (SNR), typically over 3. Given that the baseline noise is ~1 RU in the ProteOn XPR36 system, the sensorgram rise in the association phase and fall in the dissociation phase must be greater than 3 RU to guarantee the quality of the SPR results



k_a (1/Ms)	k_a Error (1/Ms)	k_d (1/s)	k_d Error (1/s)	K_D (M)	R_{max} (RU)	Chi^2 (RU^2)
1.79×10^4	2.09×10^2	4.00×10^{-2}	2.84×10^{-4}	2.23×10^{-6}	37.5	2.66

Fig. 4.23. An example of high-quality SPR results. Sensorgrams of three repeated analyte injections are overlaid and fitted together using the Langmuir model (**top**); table of fitting results (**bottom**). If the same experiment is repeated multiple times, the standard deviations of the fitting results should also be shown.

Note: The steps of data processing and data analysis using ProteOn Manager software are outlined in section 4.7.

4.6 How to Perform Excluded Volume Correction on the ProteOn XPR36 Protein Interaction System

In experiments where analytes are dissolved in a cosolvent with a high refractive index, such as DMSO, the reference surface produces a larger bulk solvent response than the ligand surface. Normally this bulk effect can be cancelled out after reference subtraction. However, the bulk effect is not equal on the interaction and reference spots. The reference surface produces a larger bulk shift/effect because of the larger concentration of cosolvent near the chip surface, caused by the exclusion by the ligand of cosolvent from the chip surface of the interaction spot. This is known as the excluded volume (EV) effect (Figure 4.24).

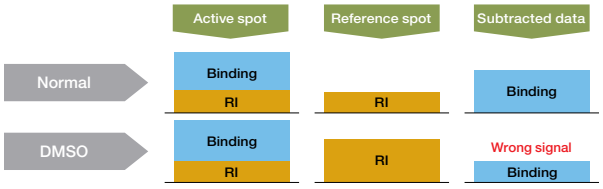


Fig. 4.24. Explanation of the EV effect when using cosolvents with high refractive index, such as DMSO. Normally the bulk effect will be cancelled out after reference subtraction. However, the bulk effect is not equal on both the active and reference spots due to DMSO exclusion by the ligand from the surface on the active channel.

Small differences in the concentration of DMSO in the analyte and running buffers also lead to large changes in response. This bulk effect is ~100 RU for every 0.1% difference in DMSO concentration.

Performing an EV correction step can cancel out these differences and lead to more reliable binding results.

To Run an Experiment With a Highly Refractive Cosolvent (DMSO)

1. Flush the instrument with ligand immobilization buffer in buffer position B. The ligand immobilization buffer usually does not contain cosolvent unless it is known not to interfere with immobilization.
2. Immobilize the ligand onto the sensor chip.
3. Determine the cosolvent concentration to be used in the experiment (for example, DMSO 5%) that will keep the analyte soluble. DMSO concentrations up to 10% are acceptable.
4. Prepare an analyte stock solution, EV calibration standards, and running buffer. These solutions should be prepared similarly to make the EV correction (EVC) the most accurate.

   For example, prepare 4–6% DMSO EVC standards, 5% DMSO running buffer, and 5% DMSO stock analyte from 10x PBS buffer as shown in Table 4.4. Dilute the stock analyte solution using the fresh 5% DMSO interaction running buffer (Figure 4.25).

Table 4.4. Preparation of DMSO solutions. Analyte and DMSO running buffer concentrations are given as an example and may change according to individual experimental needs.

	DMSO, ml 4%	DMSO, ml 6%	DMSO, ml 5%	Analyte, ml 20 µM
10x PBS	1.0	1.0	200	0.2
DMSO	0.4	0.6	100	0.08
Water	8.6	8.4	1,700	1.7
Analyte (2 mM, 100% DMSO)	—	—	—	0.02
Final Volume	10	10	2,000	2.0

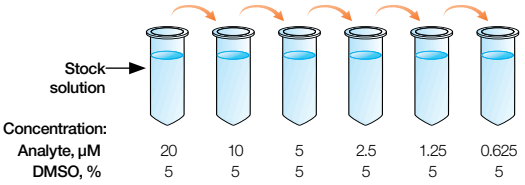


Fig. 4.25. Analyte preparation. Dilute the stock analyte solution with the highest concentration of analyte using the freshly prepared DMSO running buffer. The analyte concentration will be reduced but the DMSO concentration will stay the same (for example, DMSO 5%/PBS).

5. Flush the instrument twice with the interaction analysis buffer containing the cosolvent at the preferred concentration in buffer position A.
6. Prepare six different concentrations of DMSO in fresh running buffer (Figure 4.26).

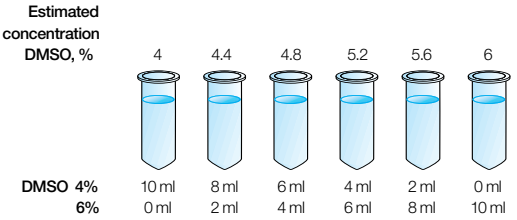


Fig. 4.26 Preparation of DMSO dilutions for EV calibration. Prepare two dilutions of DMSO in fresh running buffer, one above and one below the concentration used for the DMSO running buffer. In this example the running buffer contains 5% DMSO. Mix the two dilutions at the ratios described in the diagram to create a concentration series that has concentrations that cross over the DMSO concentration in the running buffer.

7. In the Protocol tab, after creating your protocol, click and drag the EV correction step group to the end of your protocol. The EV correction step group contains six injections by default.

Note: Blank injections that are used for double referencing must be made from the running buffer with the cosolvent.

8. Place the six DMSO dilutions into the instrument at the positions shown in the sample layout.

Processing and Applying EV Correction Data

When processing SPR data collected using a buffer that includes high refractive index cosolvents, the data's bulk reference (primary reference) must first be corrected for excluded volume effects. The data should be processed as follows:

1. Use the controls in the **Data** screen to select and group the analyte data for processing.
2. Select **Channel Reference** and choose **EVC Calibration** (Figure 4.27).

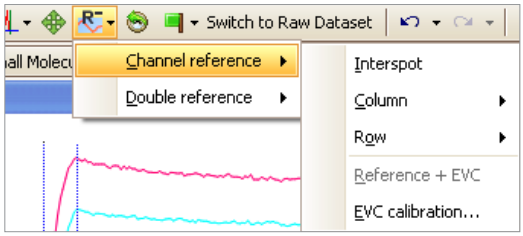


Fig. 4.27. Opening the EVC calibration wizard.

3. A wizard opens at the bottom of the **Data** screen. Select a row, column, or interspot reference. If you are using a Column or Row reference, use the associated dropdown menu to identify which channel the reference data are in.
4. In the step list, select a minimum of three EVC injections, if they are not already selected (Figure 4.28). The wizard displays EVC calibration data as thumbnail plots that show a best-fit line. These plots are accompanied by a table that lists the R^2 values for the best-fit lines.

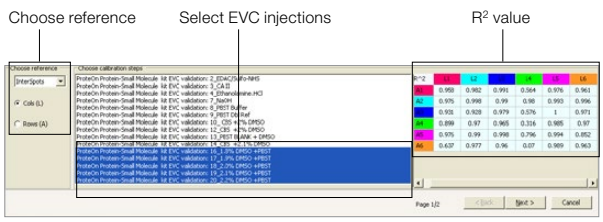


Fig. 4.28. Choosing reference and EVC injections, and viewing the R^2 value of the fit.

5. Double click the thumbnail plots with low R^2 values and then click on the bad data point to remove it. The excluded data point is represented by an empty circle and will not be included in the analysis. At least three solid data points must be selected from each calibration plot (Figure 4.29).

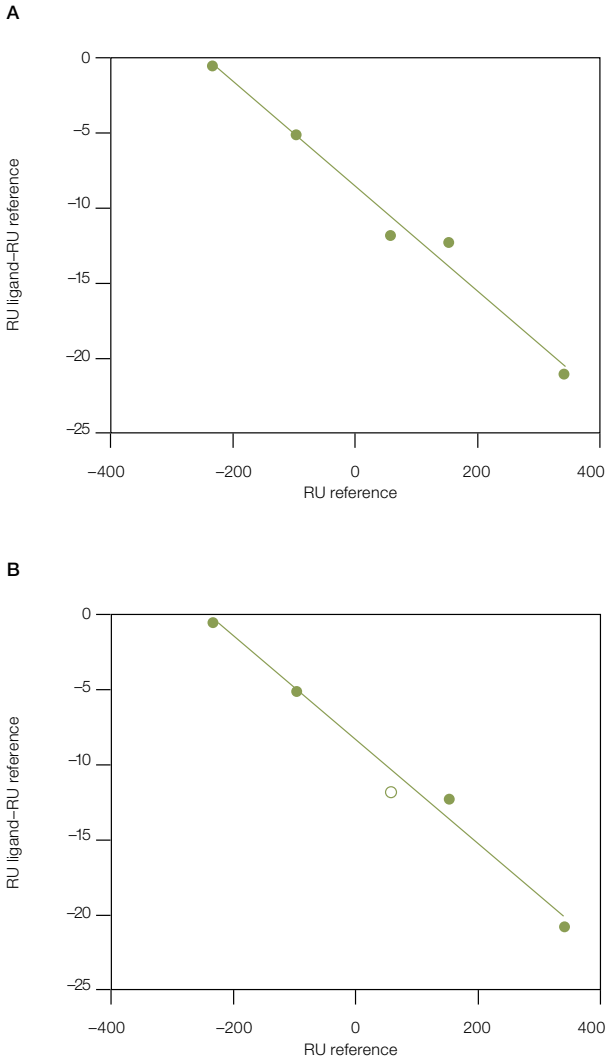


Fig. 4.29. Viewing the quality of the data for calibration. A, original data showing all five data points included in the calibration plot; **B**, modified calibration plot after removal of a data point.

6. In the second wizard step, select all the analyte steps for which you want to apply the EV correction. Click **Finish** to apply the reference and display the corrected data (Figure 4.30).

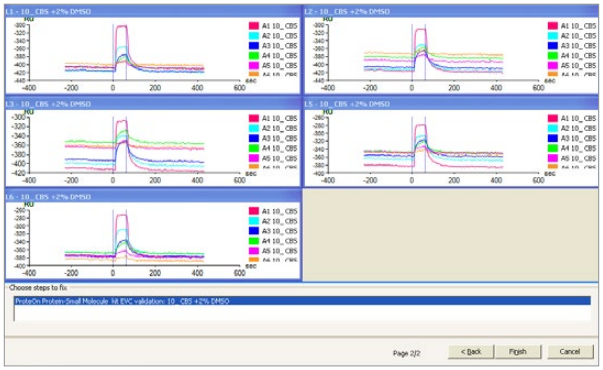
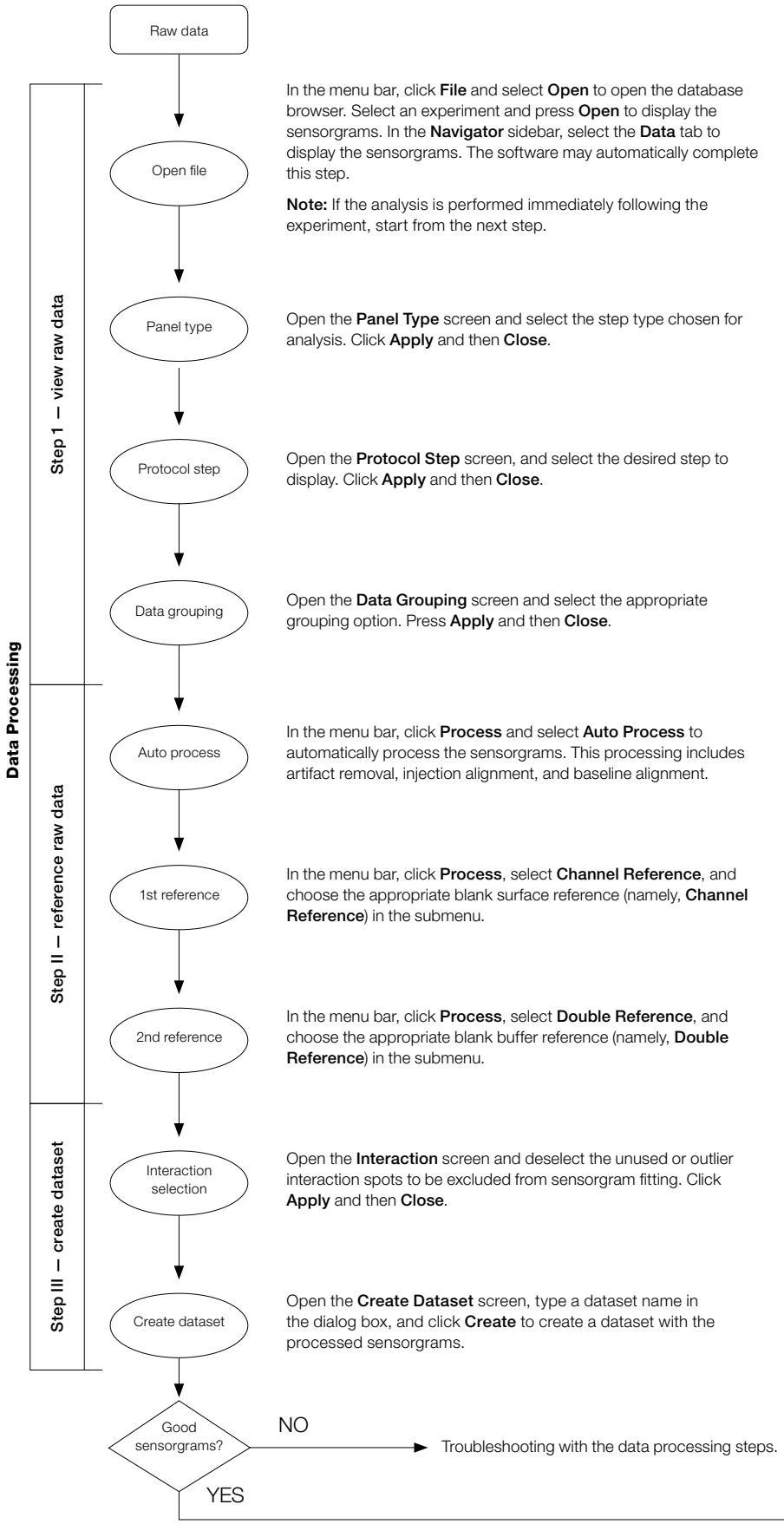


Fig. 4.30. Choosing the steps for applying EV calibration.

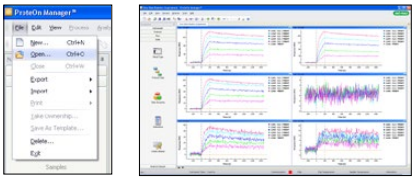
7. Apply a double reference, if desired.
8. Autoprocess the data.
9. Save the processed dataset using the **Create dataset** option.

4.7 Data Processing and Analysis Flowchart

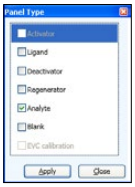


In the menu bar, click **File** and select **Open** to open the database browser. Select an experiment and press **Open** to display the sensorgrams. In the **Navigator** sidebar, select the **Data** tab to display the sensorgrams. The software may automatically complete this step.

Note: If the analysis is performed immediately following the experiment, start from the next step.



Open the **Panel Type** screen and select the step type chosen for analysis. Click **Apply** and then **Close**.



Open the **Protocol Step** screen, and select the desired step to display. Click **Apply** and then **Close**.

Open the **Data Grouping** screen and select the appropriate grouping option. Press **Apply** and then **Close**.

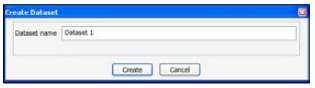
In the menu bar, click **Process** and select **Auto Process** to automatically process the sensorgrams. This processing includes artifact removal, injection alignment, and baseline alignment.

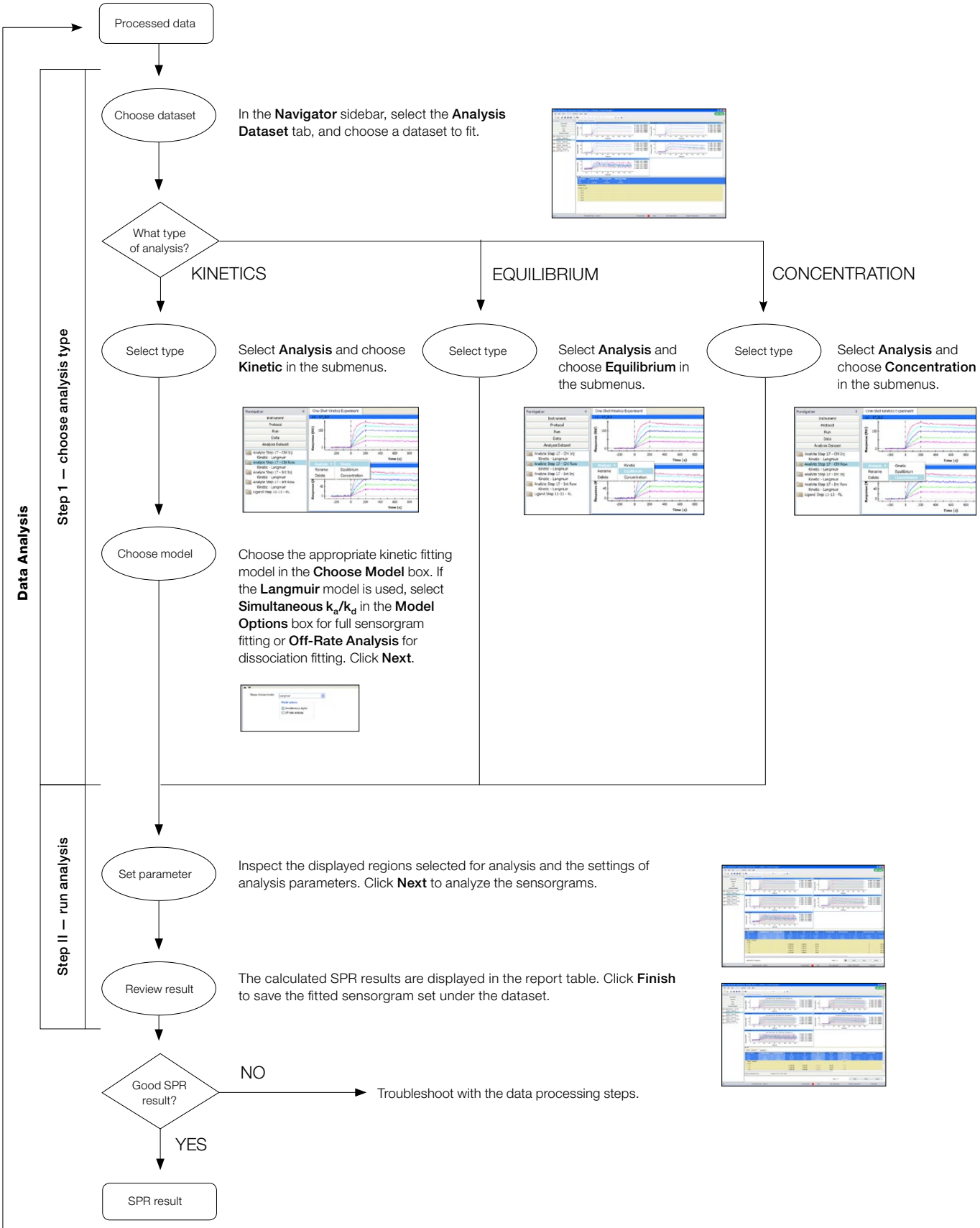
In the menu bar, click **Process**, select **Channel Reference**, and choose the appropriate blank surface reference (namely, **Channel Reference**) in the submenu.

In the menu bar, click **Process**, select **Double Reference**, and choose the appropriate blank buffer reference (namely, **Double Reference**) in the submenu.

Open the **Interaction** screen and deselect the unused or outlier interaction spots to be excluded from sensorgram fitting. Click **Apply** and then **Close**.

Open the **Create Dataset** screen, type a dataset name in the dialog box, and click **Create** to create a dataset with the processed sensorgrams.




Data Analysis
Step I — choose analysis type
Step II — run analysis
Processed data
Choose dataset
In the **Navigator** sidebar, select the **Analysis Dataset** tab, and choose a dataset to fit.
What type of analysis?
KINETICS
EQUILIBRIUM
CONCENTRATION
Select type
Select **Analysis** and choose **Kinetic** in the submenus.
Select type
Select **Analysis** and choose **Equilibrium** in the submenus.
Select type
Select **Analysis** and choose **Concentration** in the submenus.
Choose model
Choose the appropriate kinetic fitting model in the **Choose Model** box. If the **Langmuir** model is used, select **Simultaneous k_a/k_d** in the **Model Options** box for full sensorgram fitting or **Off-Rate Analysis** for dissociation fitting. Click **Next**.
Set parameter
Inspect the displayed regions selected for analysis and the settings of analysis parameters. Click **Next** to analyze the sensorgrams.
Review result
The calculated SPR results are displayed in the report table. Click **Finish** to save the fitted sensorgram set under the dataset.
Good SPR result?
NO
Troubleshoot with the data processing steps.
YES
SPR result

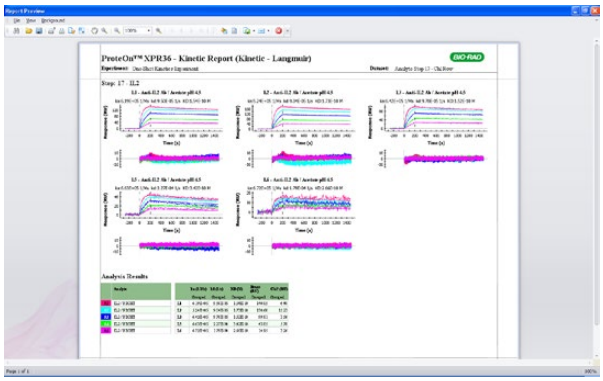
4.8 Options for Dataset Export

Three different ways of exporting a dataset are explained in the following.

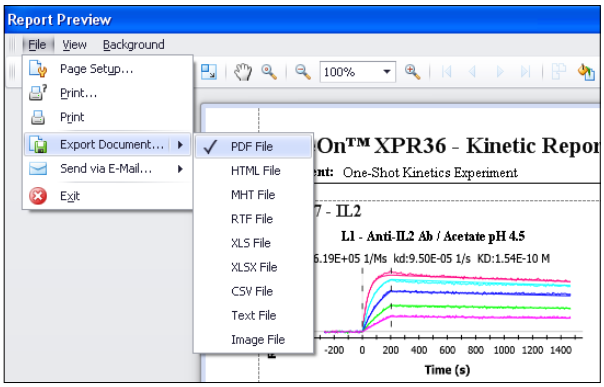
Option 1 – Print a ProteOn Manager Report

1. In the **Navigator** sidebar, enter the **Analysis Dataset** tab and choose a dataset. When a graph with overlaid fitted curves is preferred, select an analyzed sensorgram set under the dataset. If the "Unsaved processed data will be lost" dialog box appears, select **Yes**. Choose **Print** in the submenu.

2. In the **Report Options** dialog box, select the items to be included in the report and then click **OK**. The experiment report is generated in the **Report Preview** screen.



3. In the menu bar, select **File** > **Export Document** and choose the report format in the submenu. Click **OK** in the dialog box, select the target folder in which to store the report, and click **Save** to save the experiment report.

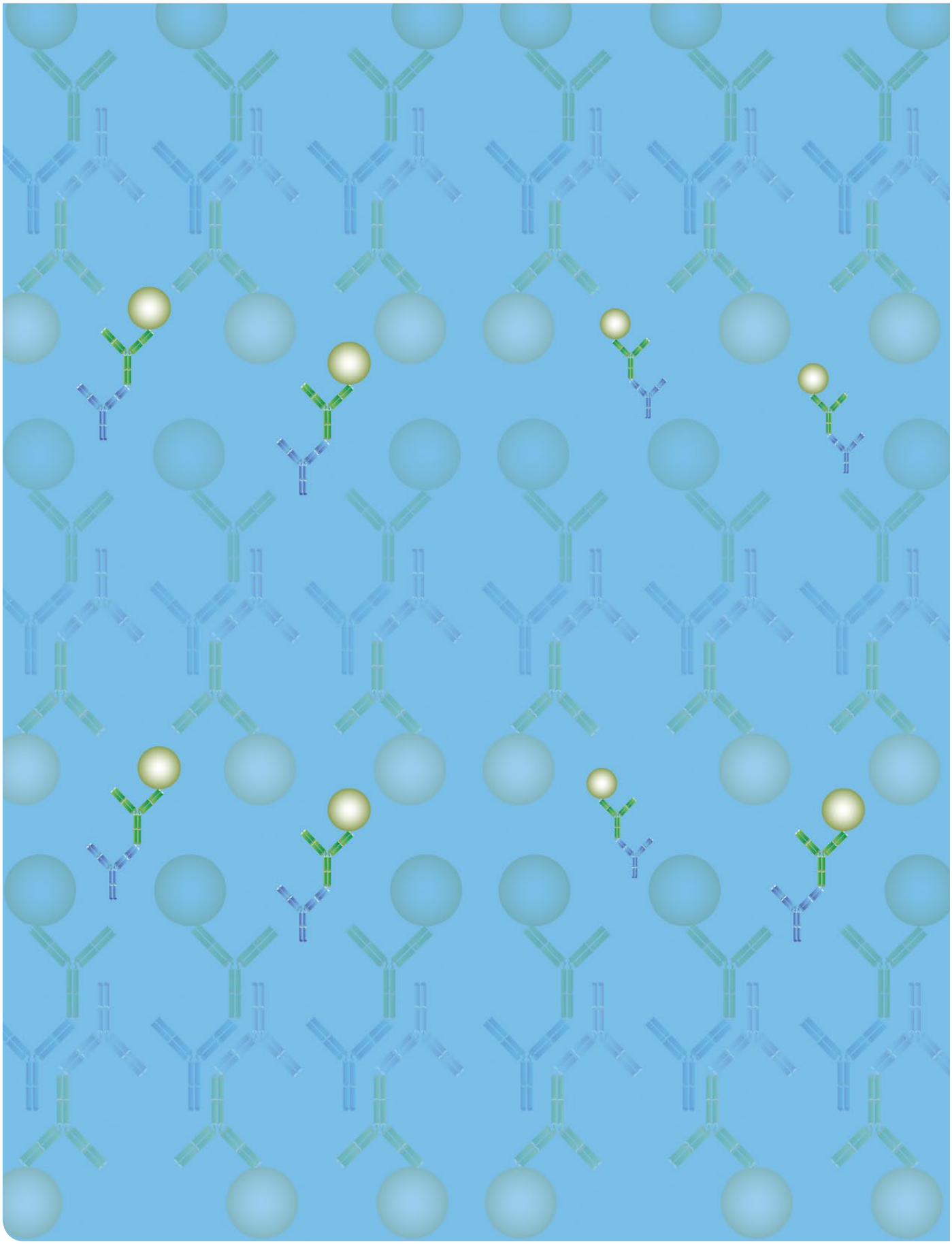


Option 2 – Copy Sensorgrams to a Presentation

1. In the **Navigator** sidebar, enter the **Analysis Dataset** tab and choose a dataset. When a graph with overlaid fitting curves is preferred, select an analyzed sensorgram set under the dataset.
2. Click to select the graph to be copied. Hold down the **Ctrl** key to select multiple graphs, or press **Ctrl+A** to select all graphs.
3. Right click any selected graph and choose **Copy Graph**. A status bar is shown. When the copy process is completed, paste the graph into the presentation.

Option 3 – Export Data to a Spreadsheet

1. In the **Navigator** sidebar, enter the **Analysis Dataset** tab and choose a dataset. If the curve-fitting data are needed, select an analyzed sensorgram set under the dataset.
2. Click to select the graph to be copied. Hold down the **Ctrl** key to select multiple graphs, or press **Ctrl+A** to select all graphs.
3. Right click any selected graph and choose **Copy Data**. A status bar is shown. When the copy process is completed, paste the graph into a spreadsheet. The first column lists time values, and the other columns list the sensorgram response values and the corresponding curve-fitting data points.



CHAPTER 5
Tips and Techniques

The tips and techniques for using ProteOn™ sensor chips, including experimental conditions and troubleshooting guides, are essential for successful surface plasmon resonance (SPR) experiments. The tips and techniques are organized by the surface chemistries of ProteOn sensor chips.

5.1 Tips for Using ProteOn Sensor Chips

All ProteOn sensor chips are designed for use with the ProteOn XPR36 protein interaction array system.

Each sensor chip is a gold-coated glass prism containing surface chemistry used for the immobilization of biomolecules (ligands) to the chip surface. This enables interactions with other biomolecules (analytes) to create a measurable SPR response used in kinetic analysis and other applications.

Refer to Chapter 2 for details on the surface chemistry of each chip.

For more information about the ProteOn XPR36 instrument and instructions for running experiments, refer to Chapters 1 and 4.

The ProteOn family of chips features outstanding performance in kinetic analysis, high binding capacities, high sensitivity for the detection of low molecular weight analytes, uniform spot-to-spot response, minimal baseline drift, barcodes, and long-term storage stability. Each ProteOn sensor chip is suitable for particular applications, including the following:

- **ProteOn GLC sensor chip** — for protein-protein interaction analysis
- **ProteOn GLM sensor chip** — for protein–small molecule and protein-protein interaction analysis
- **ProteOn GLH sensor chip** — for protein–small molecule interaction analysis
- **ProteOn NLC sensor chip** — for DNA-protein and protein-protein interaction analysis
- **ProteOn HTG sensor chip** — for protein-protein and protein-peptide interaction analysis
- **ProteOn HTE sensor chip** — for protein–small molecule interaction analysis
- **ProteOn LCP sensor chip** — for capturing lipid assemblies for lipid-protein, lipid–small molecule, and membrane protein-protein interaction analysis

The sensor chip cartridge label contains the following information:

Storing Sensor Chips

Store chips at 4°C. To avoid condensation on the chip surface, which can lead to inaccurate results, keep sensor chips in the sealed nitrogen-filled pouch during storage. Sensor chips should also remain in the pouch until reaching room temperature before use. Temperature equilibration takes from 30 to 60 min.

Opening a Sensor Chip

1. After temperature equilibration, cut the top seal of the aluminum pouch.
2. Hold the black end of the cartridge up inside the pouch to ensure the sensor chip slide remains inside the cartridge.
3. Press the sensor chip slide firmly into place within the cartridge.
4. Remove the sensor chip cartridge from the aluminum pouch.

Initializing a Sensor Chip

New sensor chips must be initialized in the instrument the first time they are used in an experiment. Follow these steps to initialize the sensor chip, using either air or glycerol.

1. Insert the temperature-equilibrated sensor chip into the instrument chip loader. The chip ID, chip chemistry, and chip expiration date populate the **Chip Details** area of the **Sensor Chip** box in ProteOn Manager™ software.
2. Choose one of the initialization options, using either air or glycerol.
3. The additional **Use Last** initialization option is available for reuse of the sensor chip. If the used sensor chip is taken out and reinserted in the instrument, glycerol initialization must be used.

Setting Up a Protocol

1. Choose **New** or **Open** from the menu bar to open the database browser.
2. Choose a **Template**, **Protocol**, or **Experiment**. Edit the name as needed for your new experiment.
3. In the **Protocol** screen, edit the configuration, samples, and protocol steps as needed.
4. In the **Instrument Control** screen, set the chip temperature and sample temperature.

5.2 Running Experiments with Sensor Chips

An interaction analysis experiment comprises five major steps:

1. Conditioning
2. Ligand immobilization
3. Stabilization
4. Analyte injection
5. Regeneration

5.2.1 Conditioning

Conditioning prepares the chip surface for use. Although optional, it is highly recommended because it can increase baseline stability. The conditioning protocols for all sensor chips are listed in Table 5.1.

Table 5.1. Conditioning parameters.

GLC, GLM, and GLH Chips				
Injection	**Reagent**	**Orientation**	**Volume, µl**	**Flow Rate, µl/min**
1	0.5% SDS	Horizontal	30	30
2	50 mM NaOH	Horizontal	30	30
3	100 mM HCl	Horizontal	30	30
4	0.5% SDS	Vertical	30	30
5	50 mM NaOH	Vertical	30	30
6	100 mM HCl	Vertical	30	30
NLC Chip				
Injection	**Reagent**	**Orientation**	**Volume, µl**	**Flow Rate, µl/min**
1	50 mM NaOH	Horizontal	30	30
2	1 M NaCl	Horizontal	30	30
3	50 mM NaOH	Vertical	30	30
4	1 M NaCl	Vertical	30	30
HTG and HTE Chips				
Injection	**Reagent**	**Orientation**	**Volume, µl**	**Flow Rate, µl/min**
1	0.5% SDS	Horizontal	30	30
2	50 mM NaOH	Horizontal	30	30
3	100 mM HCl	Horizontal	30	30
4	300 mM EDTA	Horizontal	30	30
5	0.5% SDS	Vertical	30	30
6	50 mM NaOH	Vertical	30	30
7	100 mM HCl	Vertical	30	30
8	300 mM EDTA	Vertical	30	30

Note: 1. In conditioning, it is recommended to use the same buffer used for running experiments. When working with buffers containing metal ions that form hydroxide precipitates, however, eliminate the NaOH injections. **2**. Trehalose is used as a protective layer for the dry NeutrAvidin on the NLC chip surface. It will be completely removed by continuous buffer flow over the chip surface. However, conditioning is highly recommended to ensure complete removal of the protective layer. **3**. For the LCP chip used with the ProteOn liposome capturing kit, perform conditioning after biotin-ssDNA capture and before liposome capture. For the GLC chip used with the ProteOn GLC lipid kit, perform conditioning after surface modification and before liposome capture. Refer to Section 5.2.2, Ligand Immobilization, for more details.

5.2.2 Ligand Immobilization

Ligand immobilization refers to the attachment of a ligand to the chip surface either by irreversible covalent bonding or by reversible capture using a capture reagent. Refer to Chapter 4, section 4.2 for details on achieving optimal ligand immobilization.

Desired Ligand Immobilization Level

The immobilization level (R_L), or amount of ligand immobilized on the chip surface, should be determined. The desired immobilization level is calculated using the following equation:

$$R_{max} = n\frac{M_A}{M_L}R_L$$

R_{max} is the desired maximum response when the ligand interacts with an analyte, M_A is the molecular weight of the analyte, M_L is the molecular weight of the ligand, and n is the stoichiometric coefficient of the interaction (the analyte/ligand ratio). For kinetic analysis, aiming for an analyte response with R_{max} ≤200 RU is recommended.

The approximate capacity of the amine coupling (GLX: GLC, GLM, and GLH) chips, the biotin capture (NLC) chip, and the histidine-tag capture (HTX: HTG and HTE) chips is as follows:

GLC ~8 kRU GLM ~12 kRU GLH ~20 kRU

NLC ~2 kRU HTG ~5 kRU HTE ~12 kRU

Optimizing Immobilization Conditions

In the ProteOn XPR36 system, the experimental conditions can be conveniently optimized by injecting reagents across multiple channels with each channel having different conditions, for example, varied concentrations of ligand and analyte.

GLX Sensor Chips

Activation

When using the amine coupling or GLX chips, mix 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS) to produce the activation solution. The activation reagents are typically prepared as a mixture of 1x EDC/sulfo-NHS and should be mixed immediately before the injection. It is recommended to use a contact time of 60 sec for moderate activation and 300 sec for high activation levels. For the GLH chip, avoid using a high activation level because it can lead to overactivation and subsequent multivalent linking of the ligand.

It is possible to eliminate the manual mixing of the activation reagents by using the **Co-inject** function of the ProteOn XPR36 system. Refer to the article "Ligand Immobilization in Protein Interaction Studies – An Unattended Amine Coupling Protocol with Automatic Co-Injection Activation" in the May 2012 online issue of *BioRadiations*.

Note: 1x EDC/sulfo-NHS contains a 1:1 mixture of EDC and sulfo-NHS, components that are included with the ProteOn amine coupling kit. Follow the instructions shipped with the kit to prepare the activation reagents. The final concentrations of the activation reagents are 20 mM EDC and 5 mM sulfo-NHS.

Ligand Immobilization

It is recommended to prepare the ligand in a concentration range of 0.5 µg/ml to 25 µg/ml. Typically, using a slow flow rate of 30 µl/min is suggested to reduce ligand use during ligand injection; the contact time may vary from 1 min to over 10 min, depending on the immobilization level needed. The ligand coupling buffer is ~1 pH unit lower than the ligand pI to facilitate charge attraction between the positive ligand and the negative chip surface. Low ionic strength is also required to enhance charge attraction. These conditions provide a starting point for further optimizing experimental conditions.

To achieve the desired immobilization level, choose from the two following approaches:

1. Set two consecutive ligand injection steps with a pause step in between. The first ligand injection step is short and measures the ligand immobilization rate. During the pause step, you may fine-tune the second ligand injection according to the ligand immobilization rate determined in the first step to achieve the desired immobilization level.
2. Set a single long ligand injection step and monitor the ligand immobilization process. When the desired immobilization level is reached, press the **Abort** button to end the ligand injection step.

Deactivation

Deactivation uses 1 M ethanolamine HCl at pH 8.5 to block any remaining activated carboxyl group on the chip surface. It is performed in the vertical direction, the same direction as the activation and ligand immobilization injections.

NLC Sensor Chip

Ligand Capture

It is recommended to prepare the ligand in a concentration range of 0.5 µg/ml to 25 µg/ml. Typically, using a slow flow rate of 30 µl/min is suggested to reduce ligand use during ligand injection; the contact time may vary from 1 min to over 10 min depending on the immobilization level needed. These conditions provide a starting point for further optimization of experimental conditions.

Note: The NLC chip does not need activation, and deactivation with biotin is optional.

HTX Sensor Chips

Activation and Ligand Capture

Refer to the product insert (part number 10021524) included with the HTG and HTE reagent kit for details on how to use this kit.

When using the histidine-tag capture chips or HTX chips, it is recommended to prepare the ligand in a concentration range of 0.5 µg/ml to 25 µg/ml and using a slow flow rate of 30 µl/min for the ligand injection to reduce ligand use. The contact time may vary from 1 min to over 10 min depending on the immobilization level needed. These conditions provide a starting point for the further optimization of experimental conditions. Perform ligand injection immediately after the activation step to avoid nickel (II) ion leakage and consequently a reduced immobilization level.

Table 5.2. HTX chip activation and ligand capture parameters.

Injection	Reagent	Orientation	Volume, µl	Flow Rate, µl/min
1	10 mM $NiSO_4$	Vertical	60	30
2	0.5–25 µg/ml ligand	Vertical	Flexible	30

The ProteOn HTG and HTE chips are designed to capture histidine-tagged proteins directly from crude media and purified proteins. When capturing ligand from crude samples, dilute the ligand sample before the capture to reduce nonspecific binding. It is recommended to perform a significant dilution (for example, by 100-fold), depending on the amount of active ligand in the sample.

Note: The HTG and HTE chips require activation but not deactivation. Ligand injection should be performed immediately after the activation step to avoid nickel (II) ion leakage and consequently a reduced binding level.

LCP Sensor Chip and Liposome Capturing Kit

Refer to the product insert (part number 10024332) of the liposome capturing kit for details on how to use this kit.

Activation, Conditioning, and Liposome Capture

Inject the biotin-ssDNA solution for surface activation. Then precondition the chip surface with the lipid modification conditioning solution (20 mM CHAPS) before liposome capture. Next, inject the chol-dsDNA 1 tagged liposome solution. If an additional liposome layer is needed, inject the chol-dsDNA 2 solution (0.4 µM) and allow the signal to stabilize for 5 min: then inject the chol-dsDNA 1 tagged liposome solution. Repeat this step to form multiple liposome layers.

Table 5.3. LCP chip activation, conditioning, and liposome capture parameters.

Injection	Reagent	Orientation	Volume, µl	Flow Rate, µl/min
1	1.3 µM biotin-ssDNA	Vertical	50	30
2	20 mM CHAPS	Vertical	150	30
3	1 mg/ml chol-dsDNA 1 tagged liposomes	Vertical	150	30
4	0.4 µM chol-dsDNA 2	Vertical	50	30
5	1 mg/ml chol-dsDNA 1 tagged liposomes	Vertical	150	30

Note: Injections 4 and 5 are optional.

GLC Lipid Kit

Refer to the product insert (part number 10023826) of the GLC lipid kit for details on how to use this kit.

Surface Modification

Use the activation and deactivation reagents from the ProteOn amine coupling kit. Inject 1x EDC/sulfo-NHS, lipid modification solution, and 1 M ethanolamine HCl sequentially.

Table 5.4. GLC surface modification parameters.

Injection	Reagent	Orientation	Volume, µl	Flow Rate, µl/min
1	1x EDC/ sulfo-NHS	Vertical	150	30
2	4.6 mM alkylamine	Vertical	150	30
3	1 M ethanolamine HCl	Vertical	150	30

Note: 1x EDC/sulfo-NHS contains a 1:1 mixture of EDC and sulfo-NHS. These components are included with the ProteOn amine coupling kit. It is recommended to follow the instructions shipped with the kit to prepare the activation reagents for this protocol. The final concentrations of the activation reagents are 20 mM EDC and 5 mM sulfo-NHS.

Partial Surface Modification

The extent of surface modification can be used to control the liposome capture level and configuration. Lower surface modification will reduce the capture capacity and in many cases improve the resistance to nonspecific binding. Lower modification will also increase the chances of capturing intact liposomes, while higher modification will increase the tendency of liposomes to deform and even open into lipid bilayers spread over the surface.

Partial surface modification refers to varying the immobilization level of undecylamine by controlling the immobilization conditions. This can be achieved by controlling the activation level, undecylamine concentration, or injection volume. Otherwise, mixing undecylamine with ethanolamine HCl (the deactivation solution from the amine coupling kit) is an easy and efficient alternative. For fine-tuned control of the surface modification level, it is recommended to vary the undecylamine concentration by diluting the lipid modification solution (undecylamine) in the lipid modification conditioning solution (CHAPS).

Liposome Capture

Before liposome capture, condition the chip surface with the lipid modification conditioning solution (20 mM CHAPS), and then inject the liposome solution.

Table 5.5. Liposome capture parameters.

Injection	Reagent	Orientation	Volume, µl	Flow Rate, µl/min
1	20 mM CHAPS	Vertical	150	30
2	1 mg/ml liposomes	Vertical	150	30

When injecting ligand, concentrations between 0.5 and 25 µg/ml are typically used with a flow rate of 30 µl/min and a contact time of 1–14 min. These conditions will produce signals of up to 5,000 RU on the HTG chip and 12,000 RU on the HTE chip.

Please note that when capturing ligands from crude media, various other proteins may also be adsorbed to the chip surface, mainly due to nonspecific interaction with nickel (II) ions. Fortunately, these proteins will not interfere with the kinetics because ligand-analyte interactions are specific.

However, the presence of nonspecifically bound lysate proteins will make accurate determination of ligand binding levels difficult given that the observed signal is the sum of the ligand signal and the signal from other bound proteins.

5.2.3 Troubleshooting Ligand Immobilization

Amine Coupling with GLX Chips

The degree of success in ligand immobilization can be visualized by observing the sensorgram during the procedure (see Figure 5.1).

Typical Ligand Buffer Conditions

The typical ligand buffer conditions are listed below:

1. The pH should be one unit below the pI value.
2. The ligand buffer ionic strength should be low, and the ligand concentration should be above 0.1 µg/ml.
3. If a disulfide bond reduction reagent is needed in the ligand buffer, TCEP is preferred over DTT because TCEP is compatible with the amine coupling protocol.

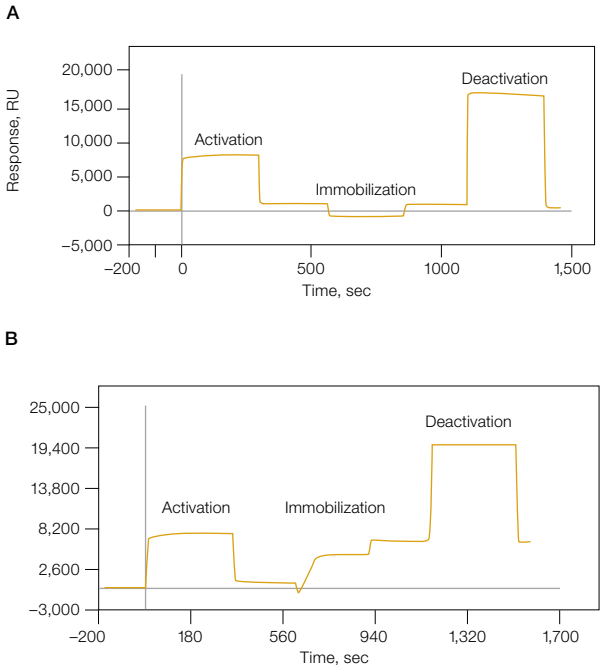


Fig. 5.1. Sensorgram examples of failed (A) and successful (B) ligand immobilization by amine coupling.

Activation Quality

Verify the activation quality by:

1. Using fresh activation reagents.
2. Immobilizing another protein that was previously used under the same conditions with success.

Ligand Activity

If a sufficiently high R_{max} is predicted based on the R_L value, but the binding response is much lower than the expected response, this indicates low ligand activity on the surface. Because the amine coupling method randomly links any free amine group on the ligand molecule to the chip surface, the ligand activity is usually not very high.

If low ligand activity is caused by incorrect ligand orientation in amine coupling, there are multiple alternative ligand immobilization methods typically used as solutions.

1. **Capture surface** — use a chip surface functionalized with some capture agent such as antibody, biotin-binding proteins (avidin family proteins), histidine-tag-binding reagents, etc. It should be noted that the ligand must be biotinylated or histidine-tagged to use biotin or histidine-tag binding surface chemistry (Figure 5.2A).
2. **Protected immobilization** — premix the ligand with a known reagent binding to the analyte-binding site, for example premix kinase (ligand) with a known inhibitor in the case of screening new inhibitive compounds. The ligand-reagent complex formed in the solution guarantees the right orientation of the ligand when it is immobilized on the chip surface. The ligand surface is then regenerated and ready for the ligand-analyte interaction analysis (Figure 5.2B).
3. **Aldehyde coupling** — if the ligand contains aldehyde groups not located at the analyte-binding site, aldehyde coupling can be used to increase the ligand activity. It is typically used to increase the activity of human antibody, in which case the polysaccharide side chain of an antibody is oxidized by $NaIO_4$ (sodium peroxide) to create aldehyde groups. Aldehyde coupling can be achieved on GLX chips by sequential injections of the reagents found in Table 5.6.

Table 5.6. Aldehyde coupling on GLX chip.

Injection	Reagent	Reaction to Chip Surface
1	EDC/sulfo-NHS	Activation of carboxyl groups
2	Carbohydrazide	Creation of amine groups
3	Ethanolamine	Deactivation of carboxyl groups
4	Ligand	Immobilization of ligand by Schiff base reaction
5	$NaB(CN)H_3$ (sodium cyanoborohydride)	Stabilization of ligand by reduction of Schiff base

4. **Thiol coupling** — if the ligand contains thiol groups not located at the analyte-binding site, thiol coupling can be used to increase the ligand activity. It is typically used when the ligand contains cysteine residues far from the analyte-binding site. Thiol coupling can be achieved on GLX chips by sequential injections of the reagents in Table 5.7.

Table 5.7. Thiol coupling on GLX chip.

Injection	Reagent	Reaction to Chip Surface
1	EDC/sulfo-NHS	Activation of carboxyl groups
2	Cysteine	Creation of thiol groups
3	Ethanolamine	Deactivation of carboxyl groups
4	DTNB (5,5'-dithiobis-(2-nitrobenzoic acid))	Formation of disulfides
5	Ligand	Immobilization of ligand by substitution of disulfides

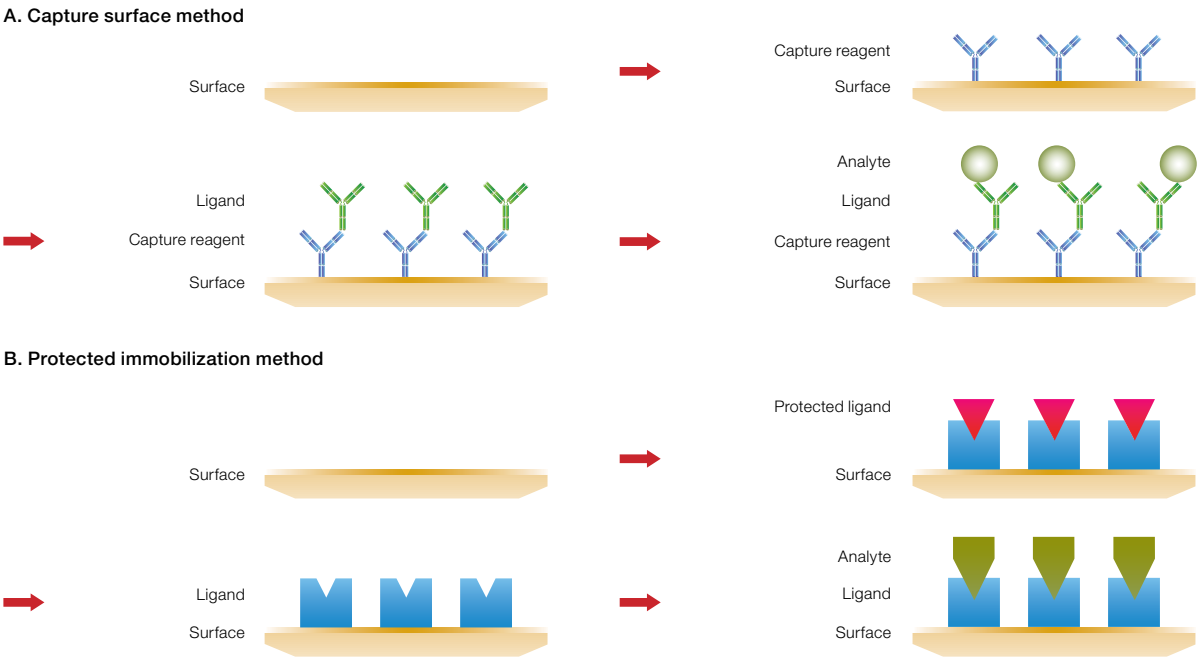


Fig. 5.2. The capture surface method (A) and protected immobilization method (B) for higher ligand activity.

Ligand Capture with the NLC Chip

Tips for capturing ligands with NLC chips:

1. It is important to assess the result of biotinylation after ligand preparation. If the biotinylation step was not successful, ligand capture will not be observed on NLC chips.
2. If excess biotin is not removed properly from the ligand sample, it will occupy the available binding sites on the chip surface and result in low binding levels, typically of a few tens of RU.
3. If the ligand is biotinylated and captured on the chip surface but does not show a binding response with the analyte, over-biotinylation may have occurred. To avoid this, prepare the ligand in a stoichiometry of 1:1 (one biotin molecule per ligand molecule). This also prevents cross-linking of the ligand. Alternatively, carry out the biotinylation reaction in a low pH buffer (50 mM acetate at pH 5.5) to favor the selective biotinylation of alpha-amino groups, which leaves the lysine residues unblocked.

5.2.4 Stabilization

Stabilization is the step between ligand immobilization and analyte injection. Injecting running buffer or regeneration solutions removes any noncovalently bound ligand molecules from the chip surface. Thus, stabilization creates the stable baseline required to perform the interaction analysis.

Stabilization involves performing one or more injections of either the running buffer or regeneration solutions that do not affect the immobilized ligand. Allowing the baseline to stabilize for 30 min is recommended to obtain high-quality kinetic analysis. For the NLC chip, it is recommended to inject 1 M NaCl in the stabilization step.

5.2.5 Analyte Injection

In the ProteOn XPR36 system, ligands and analytes are typically injected at perpendicular directions in the 6 x 6 configuration to perform the interaction analysis. This patented One-shot Kinetics™ approach allows up to 36 individual interactions to be performed simultaneously in a single analyte injection, providing high efficiency in experiment optimization and high throughput in data production. Ligands and analytes are typically injected in the vertical and horizontal directions, respectively. For kinetic analysis, analyte injections are usually performed at a high flow rate (for example, 100 µl/min), but a lower flow rate may be used to reduce sample consumption. The injection conditions, including association and dissociation time, flow rate, and analyte concentrations, should be optimized to obtain high-quality interaction analysis. Refer to Chapter 4, section 4.3 for further details on achieving optimal ligand-analyte interaction analysis. Three useful tips for obtaining reliable interaction analysis results are listed below:

1. Prepare the analyte samples as a concentration series, typically a two- or threefold dilution series centered around the expected K_D.
2. If needed, set up a double reference. Replace one of the six analyte channels with running buffer for use as a real-time double reference (row reference). Alternatively, set up an injection of running buffer into all six analyte channels prior to the injection of analyte samples (injection reference). Double referencing is needed for baseline drift correction and is used mostly when the ligand is reversibly captured by a capture reagent such as an antibody, NeutrAvidin (NLC and LCP chips), or a tris-NTA complex (HTG and HTE chips).
3. Set the association time of the interaction to be long enough to observe curvature in the association phase. Similarly, allow the dissociation time of the interaction to be long enough to observe a signal drop in the dissociation phase.

The concentration range of analyte should span 10x above and 10x below the expected K_D. A buffer blank can also be injected as a real-time double (row) reference.

Note: In protein–small molecule interaction analysis, the small molecule analyte is sometimes prepared with a high refractive index cosolvent such as DMSO. In such a case, excluded volume correction is typically used to account for the excluded volume effect while maintaining the accuracy of referencing. Refer to Chapter 4, section 4.6 for details on how to apply this correction.

Troubleshooting Analyte Injection

Nonspecific Binding

Nonspecific binding (NSB) is defined as the direct binding of an analyte or sample components other than the target ligand to the sensor chip surface. NSB is characterized by significant binding responses that occur on reference spots and do not return to baseline at the end of the injection (Figure 5.3). These events can potentially skew experimental results. In theory, if the NSB responses on the interaction surface and on the reference are similar, subtracting the reference from the interaction response will correct the data and lead to accurate fitting to the binding model. In practice, however, it is very difficult to determine whether NSB is similar on the interaction and reference surfaces. There are cases where the ligand molecules on the interaction surface block NSB on the chip surface. This leads to a higher NSB response on the reference surface and results in incorrectly referenced data or even negative responses.

At present, NSB is one of the most difficult factors to optimize in label-free biomolecular interaction analysis. There are two main strategies used in SPR biosensors to overcome NSB:

1. Using a reference surface that is as similar to the ligand surface as possible. Optimally, a reference protein unrelated to the ligand should be bound at the same density as the ligand.
2. Minimizing NSB by optimizing the buffer conditions and surface chemistry.

Electrostatic NSB

NSB is most commonly caused by the electrostatic attraction of a positively charged analyte or other sample components to the negatively charged surface layer of the sensor chip (Figure 5.4). This type of NSB, termed electrostatic NSB, is common when the analyte is a protein with a pI higher than the pH of the running buffer.

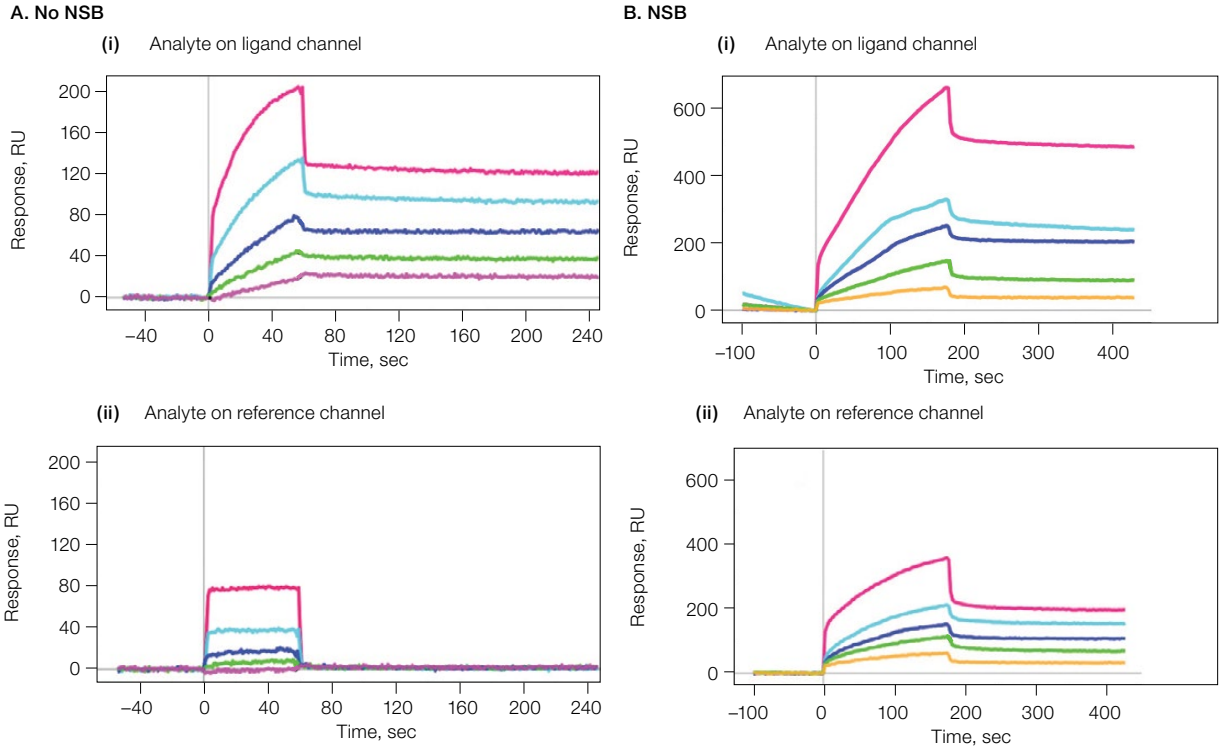


Fig. 5.3. Comparison of responses on reference surfaces showing only bulk effect (a refractive index difference between the sample buffer and running buffer) (A) or exhibiting NSB (B). A (ii), for the analyte injected in the reference channel showing no NSB, the analyte response is flat during the injection and returns to zero at the end of the injection. **B (ii)**, for the analyte injected in the reference channel showing NSB, the analyte response exhibits curvature during the injection and does not return to zero at the end of the injection. RU, response units.

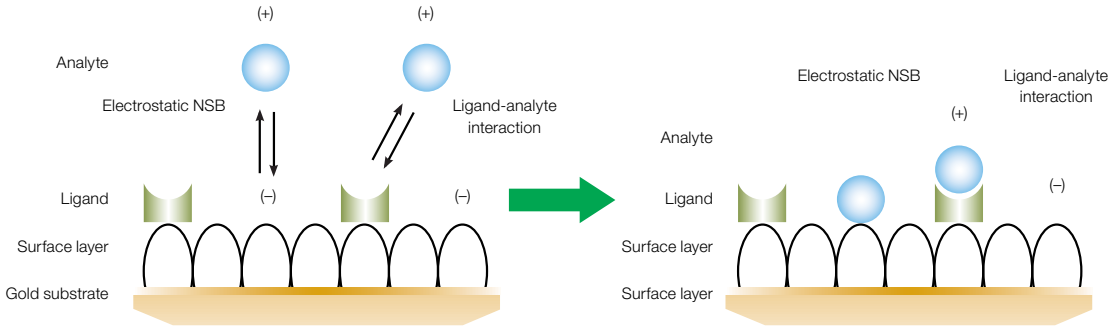


Fig. 5.4. Electrostatic NSB on the chip surface.

Non-Electrostatic NSB

Sources of NSB also include chemical interactions of the analyte or other components with the binding layer, such as hydrophobic interactions, hydrogen bonding, or binding to nanoscopic areas of exposed gold on the surface (Figure 5.5). This type of NSB, termed non-electrostatic NSB, is usually observed when "sticky" or crude analyte samples are applied. When using the HTG or HTE chips, non-electrostatic NSB can also be caused by proteins with a sequence containing a few adjacent histidine residues, which have a low affinity for the nickel (II)-activated tris-NTA surface. Molecules that can potentially exhibit electrostatic and non-electrostatic NSB are listed in Table 5.6.

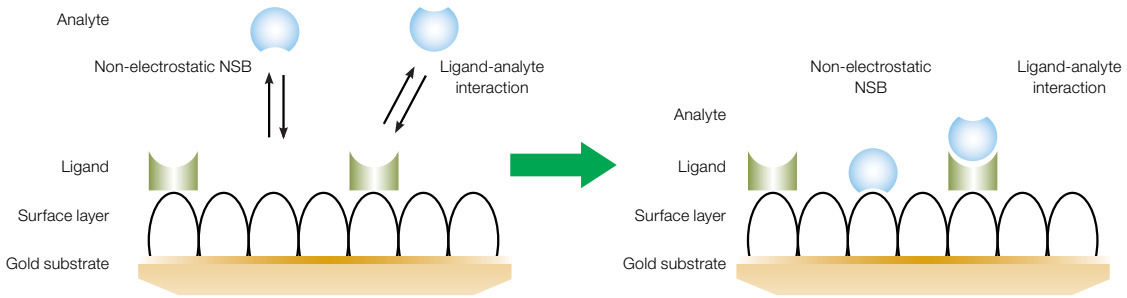


Fig. 5.5. Non-electrostatic NSB on the chip surface.

Table 5.6. Molecules with a propensity for NSB.

Molecules in Samples	Type of NSB
Positively charged proteins with a pI > pH of the running buffer	Electrostatic NSB
Relatively small molecules containing thiol groups or related forms of sulfur, such as cysteine-containing peptides, known to show high affinity to gold	Non-electrostatic NSB
Molecules that naturally bind polysaccharides, such as lectins, may exhibit NSB to the alginate-containing binding layer	Non-electrostatic NSB
Biological solutions such as serum, crude lysates, or supernatants	Electrostatic NSB and Non-electrostatic NSB

Techniques for Reducing or Eliminating NSB on All Sensor Chips

Suggested changes in experiment design to reduce NSB:

1. Optimize the appropriate running buffer and sample buffer for the application.
2. Purify the analyte or sample when possible.
3. Try different immobilization surface chemistries; for example, use a biotinylated ligand.
4. Swap the ligand and the analyte if only the analyte shows NSB.
5. Retest your binding assay with a fresh chip, preferably from a different lot, to eliminate chip-specific defects.

Table 5.7. Techniques for reducing or eliminating NSB on all sensor chips.

Methods	Details	Non-electrostatic NSB	Electrostatic NSB
Increase the buffer salt concentration	Increase the salt concentration to up to 500 mM in the analyte buffer and running buffer, using NaCl for example, to shield the electrostatic charges. It is important to verify that the high salt concentration does not affect the ligand or analyte activity.	—	•
Increase the buffer pH	Increase the pH of the analyte buffer and running buffer to reduce the positive charges contributing to electrostatic NSB. It is important to verify that the high pH does not affect the ligand or analyte activity.	—	•
Add 0.05% Tween 20 and/or 0.1% BSA	Add 0.05% Tween 20 and/or 0.1% BSA to the running buffer to reduce both electrostatic and non-electrostatic NSB; 0.1% BSA may also be used to saturate the chip surface to block potential NSB sites.	•	•
Create an appropriate reference surface	Create an appropriate reference surface by capturing a reference protein unrelated to the ligand, such as BSA, to the same level as the ligand. The reference protein does not reduce NSB directly, but it shields the charges on the chip surface as much as the ligand. NSB will be corrected for by subtracting the reference.	—	•
Dilute the analyte	When using a complex analyte sample like serum or crude lysate, dilute the sample with the running buffer — a five- to tenfold dilution is usually recommended. Higher dilution rates should be used if the sample is very concentrated.	•	•
Use prepurification	Use simple prepurification methods to remove the majority of the contaminants in the analyte sample.	•	•
Remove albumin from the ligand sample	If a ligand sample contains albumin, inject a pulse of 1 M salt solution to remove the albumin bound to the chip surface before the analyte injection.	•	•

Note: Refer to Bio-Rad bulletin 6302 for specific troubleshooting tips for HTX chips.

Bulk Effect

The bulk effect refers to a spurious SPR response that is caused by changes in the refractive index of the solution near the sensing surface rather than the binding of biomolecules to the surface. Such refractive index changes typically occur during the sequential injection of two solutions with different compositions such as different salt, detergent, or biomolecule concentrations. A small bulk effect can be completely removed by applying proper referencing, but a large bulk effect may cause inaccuracy in data processing and analysis. To minimize the bulk effect, the refractive index of the running buffer should match that of the sample buffer.

Correct for Bulk Effect

1. Apply proper referencing.
2. Match the refractive index of the sample buffer and running buffer.
3. In experiments where analytes are dissolved in a cosolvent with a high refractive index, such as DMSO, the reference surface produces a larger bulk solvent response than the ligand surface because of the larger concentration of cosolvent near the reference surface. This effect is due to the exclusion of cosolvent by the ligand near the ligand surface. The resulting difference in bulk effect causes inaccurate reference subtraction. To correct for the difference in bulk effect between interaction and reference surfaces, excluded volume correction is applied; refer to Chapter 4, section 4.6 for more details.

Bubble Formation

Separation air bubbles are intentionally created between the sample and running buffer to prevent mixing during sample aspiration. No bubbles should be injected into the ligand or analyte channels. Spikes in the sensorgram usually indicate the injection of bubbles. Small spikes can be completely removed in sensorgram processing, but large spikes may cause inaccuracy in experimental results.

Problems with bubbles during the injection step:

1. Bubbles at the beginning of an injection are usually coupled with an injection delay in one or more channels.
2. Responses may vary in intensity among different channels, injections, and times.

Techniques for Reducing Bubble Formation

1. Use prepierced vial caps and microplate sealing films to prevent vacuum formation inside the container during aspiration.
2. Degas the sample and reagent solutions.
3. Verify that the volumes of sample and reagent solutions are sufficient before injection.

5.2.6 Regeneration

Regeneration removes the analyte or ligand-analyte complex on the chip surface to prepare the surface for the next experiment without damaging the ligand and/or chip surface. Regeneration conditions should be optimized for each interaction. The reproducibility of repeated analyte injections is typically used to check the performance of regeneration. With a good regeneration protocol, the sensorgrams of repeated analyte injections should overlap when viewed in the same window.

GLX and NLC Sensor Chips

For GLX and NLC chips, the ligand is bound to the chip surface by covalent or very high affinity noncovalent linking. Regeneration is used to remove the analyte while keeping the ligand active on the chip surface. The regeneration conditions should be optimized to a balance that is strong enough to completely remove the analyte but not so harsh as to damage the ligand. Some recommended conditions for different interaction systems are listed below.

Table 5.8. GLX and NLC chip regeneration reagents.

Ligand	Analyte	Recommended Reagent
Protein/antibody	Protein/peptide	10 mM glycine pH 1.5–3.0, 1% phosphoric acid
Peptide/ nucleic acid	Protein/peptide	0.01–0.5% SDS/ 5–10 mM NaOH
Nucleic acid	Nucleic acid	5–10 mM NaOH/ deionized water

HTX Sensor Chips

The captured ligand can be stripped off and replaced with fresh ligand by a highly efficient regeneration step using 300 mM EDTA, pH 8.5. Once the ligand is removed, the chip can be reactivated to capture new ligands.

Table 5.9. HTX chip regeneration conditions.

Injection	Reagent	Orientation	Volume, ul	Flow Rate, ul/min
1	300 mM EDTA, pH 8.5	Vertical	400	30

EDTA may not completely remove nonspecifically adsorbed proteins because they are adsorbed to the surface not only via the nickel (II) ions but, for example, also by electrostatic interactions. In such cases, other ProteOn regeneration solutions, such as 50 mM NaOH and 100 mM HCl, may be needed along with EDTA to regenerate the surface.

LCP Chip and Liposome Capturing Kit

Regeneration is accomplished by DNA dehybridization using the following conditions. Injection 2 is optional because it is used to remove the remaining lipid assemblies if the regeneration is incomplete with injection 1. For the first injection, use a freshly prepared 8 M solution of urea in deionized water.

Table 5.10. LCP chip regeneration conditions.

Injection	Reagent	Orientation	Volume, ul	Flow Rate, ul/min
1	8 M urea*	Vertical	150	30
2	20 mM CHAPS	Vertical	150	30

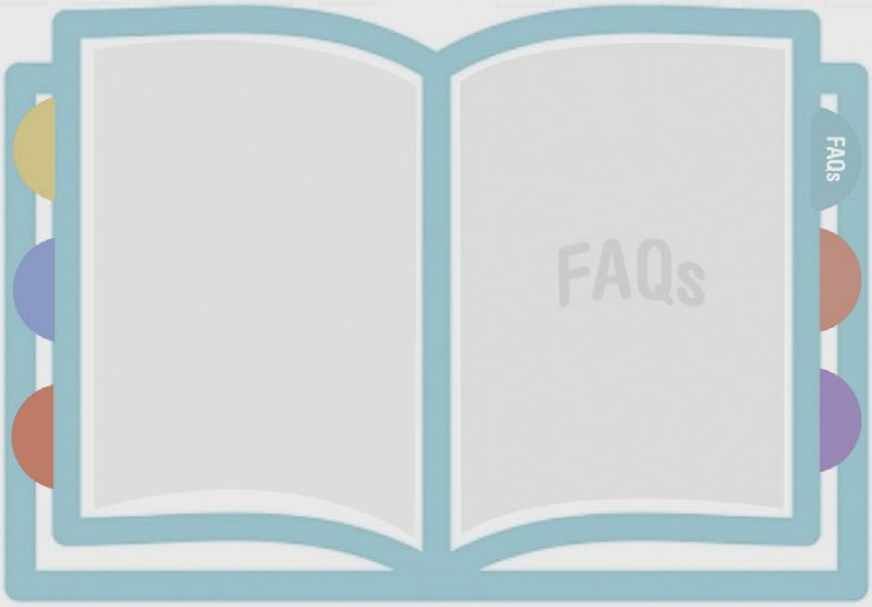
***** If urea is not available, inject deionized water.

GLC Lipid Kit

When using the GLC lipid kit, injecting lipid modification solution (20 mM CHAPS) will regenerate the chip surface.

Table 5.11. GLC chip regeneration conditions.

Injection	Reagent	Orientation	Volume, ul	Flow Rate, ul/min
1	20 mM CHAPS	Vertical	150	30


FAQs
FAQs

CHAPTER 6
Frequently Asked Questions

Answers are provided to frequently asked questions on how to use the ProteOn™ XPR36 system.

6.1 Basics

What is SPR? What does SPR measure?

SPR is surface plasmon resonance. It senses the refractive index change (mass change) within a thin layer on the surface of a metal that is in contact with a dielectric medium.

What applications can the ProteOn XPR36 system be used for?

- Real-time kinetic analysis of biomolecular interactions
- Equilibrium analysis for affinity constant determination
- Protein concentration quantitation

What do the SPR terms sensor chip surface, ligand, analyte, and capture reagent mean?

Sensor chip surface (or surface) — a metal surface coated with a polymer where SPR is measured. The surface is located on an SPR sensor chip.

Ligand — an interaction reagent immobilized on the surface, also often referred to as the target.

Analyte — an interaction reagent flowed over the ligand immobilized to the surface.

Capture reagent — a reagent immobilized to the surface that is used to capture the ligand by biological interactions. It is used to reversibly immobilize the ligand to the surface.

6.2 Sensorgram

What are the descriptors used to define specific regions of an SPR sensorgram? Which regions are used to calculate the kinetic and equilibrium constants k_a, k_d, and K_D?

The association phase shows second-order kinetics and the dissociation phase shows first-order kinetics. The regions used to calculate the constants are shown in the graph (Figure 6.1).

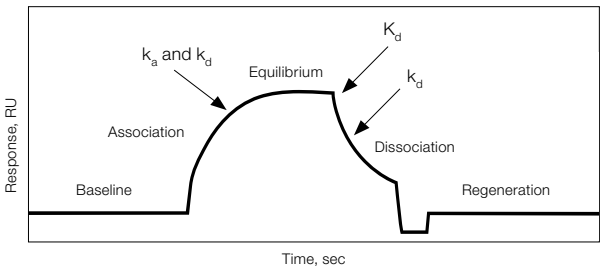


Fig. 6.1. Descriptors of an SPR sensorgram and regions to calculate the kinetic and equilibrium constants. RU, response units.

In an SPR sensorgram, which parameters are necessary for the calculation of kinetic constants?

For the association kinetic constant (k_a), the analyte concentration is necessary for the calculation. For the dissociation kinetic constant (k_d), no parameter is needed.

6.3 Sensor Chips

What types of sensor chips does Bio-Rad offer?

ProteOn GLC sensor chip — for general amine coupling, polymer matrix layer with compact binding capacity of approximately one protein layer.

ProteOn GLM sensor chip — for general amine coupling, polymer matrix layer with intermediate binding capacity.

ProteOn GLH sensor chip — for general amine coupling, polymer matrix layer with high binding capacity.

ProteOn NLC sensor chip — for capturing biotinylated molecules, polymer matrix layer containing NeutrAvidin with compact binding capacity.

ProteOn HTG sensor chip — for capturing histidine-tagged proteins, polymer matrix layer containing tris-NTA complexes with compact binding capacity.

ProteOn HTE sensor chip — for capturing histidine-tagged proteins, polymer matrix layer containing tris-NTA complexes with high binding capacity.

ProteOn LCP sensor chip — for capturing lipid assemblies such as liposomes, for use with LCP capturing reagent kit.

On a single SPR sensor chip, can I use one spot/channel at a time for ligand immobilization and reserve the blank spots/channels for a future experiment?

Yes, it is possible to immobilize ligands in individual spots/channels with any of the amine coupling, histidine-tag capture, and lipid assembly capture sensor chips. This may not apply to streptavidin or NeutrAvidin sensor chips if an additive is used to stabilize these proteins on the surface.

Can I reuse a spot/channel after a ligand is immobilized to the surface of the sensor chip?

It is possible to reuse a spot/channel after a ligand has been immobilized to the surface of the sensor chip, if the chip surface is regenerated and preserved properly to keep the ligand activity.

How many times is it possible to regenerate a sensor chip surface?

The extent of regeneration depends on the immobilized protein ligand. In the case of small molecule screening, running buffer is often the regeneration solution. Therefore, the protein ligand remains quite stable and active for multiple rounds of analyte injections. However, when an acid, base, or detergent is required for regeneration, the ligand may lose its activity and a positive control is recommended to monitor the ligand activity.

Is the ProteOn XPR36 system able to screen small molecules? Which sensor chip is suggested for analyzing small molecules?

Yes. The GLH chip has high ligand binding capacity in the amine coupling chips, and the HTE chip has high capacity for capturing histidine-tagged targets. They are both designed for this type of analysis.

Are glycoproteins compatible with the ProteOn sensor chips?

Glycoproteins can be immobilized using amine coupling or captured on an NLC chip if the glycoprotein is biotinylated.

What are the differences between NTA and tris-NTA surface chemistry in capturing histidine-tagged proteins?

The nickel(II)-activated nitrilotriacetate (NTA) surface chemistry is often used for capturing histidine-tagged proteins in SPR experiments. Tris-NTA (3 x NTA) surface chemistry is derived from NTA with improved capture stability and selectivity. It results in minimal ligand drift and improves sensorgram baseline stability. Compared to NTA sensor chips, the tris-NTA sensor chips allow easy surface regeneration, chip reuse, and capture of histidine-tagged proteins directly from crude samples. The Bio-Rad ProteOn HTG and HTE sensor chips are tris-NTA sensor chips.

What are the applications of ProteOn HTG and HTE sensor chips?

The HTG and HTE sensor chips feature a novel tris-NTA surface for improved capture of histidine-tagged proteins. The functional group, tris-NTA, is unique to the ProteOn HTG and HTE sensor chips and has a significantly higher binding stability compared to that of the traditional NTA surface (Figure 6.2). Bio-Rad offers two ProteOn sensor chips for various histidine-tagged protein applications: HTG for compact-density (large molecule) applications and HTE for high-density (small molecule) applications. Both the HTG and HTE sensor chips allow easy surface regeneration, chip reuse, and capture of histidine-tagged proteins directly from crude samples.

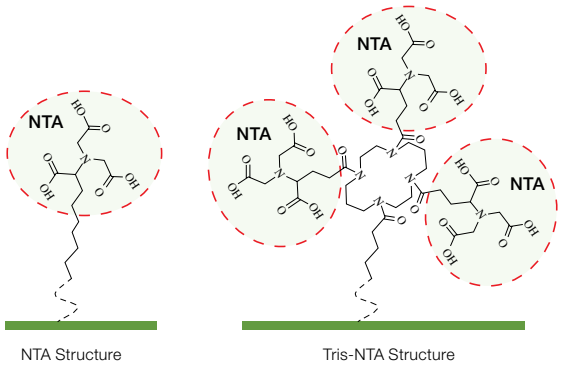


Fig. 6.2. Structures of NTA and tris-NTA bound to SPR sensor chips.

The challenge in working with membrane proteins is finding methods to capture the membrane proteins while keeping them active. Is there any information about the study of membrane proteins with the ProteOn XPR36 system?

If the soluble form of membrane proteins is available for use, you may immobilize the membrane proteins with the same methods as for other protein targets, such as amine coupling and antibody capture. However, many membrane proteins require a lipophilic environment to maintain the ability to react with biomolecules. A common method of maintaining lipophilic environments is to embed proteins in lipid assemblies such as liposomes.

Biomolecular interactions involving lipid assemblies such as liposomes is an interesting direction in biological research today. An essential purpose of using lipid assemblies is attaining native membrane proteins embedded in the lipid bilayer of these assemblies, by which the activity of the membrane proteins is maintained. In order to facilitate the interaction analysis with lipid assemblies, two ProteOn kits have been developed: GLC lipid kit and the liposome capturing kit. These kits facilitate

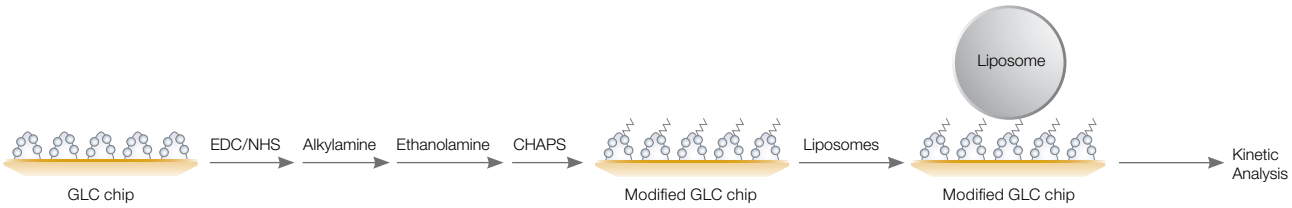


Fig. 6.3. Workflow for liposome capture using the ProteOn GLC lipid kit, based on the traditional lipophilic surface chemistry. The lipophilicity of the GLC chip surface is adjusted through surface modification in order to capture lipid assemblies such as liposomes.

membrane-involved interactions analysis such as lipid-protein interactions,
lipid–small molecule interactions, and membrane protein-protein interactions, which are usually considered difficult targets in label-free interaction analysis.

ProteOn GLC lipid kit — this kit is based on the traditional approach of capturing liposomes using a modified lipophilic GLC sensor chip surface (Figure 6.3). It also provides the flexibility to adjust the surface chemistry for a particular application.

ProteOn liposome capturing kit — This kit includes a new LCP sensor chip that is designed for use with the ProteOn LCP capturing reagent kit (Figure 6.4). This kit provides a novel hydrophilic surface chemistry that allows for advanced applications, such as minimizing lipophilicity-based nonspecific binding and capturing liposomes that are difficult to analyze with the traditional approach. It is possible to capture multiple layers of lipid assemblies for additional sensitivity.

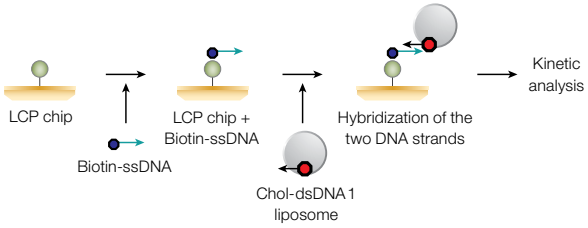


Fig. 6.4. Workflow for liposome capture using the ProteOn liposome capturing kit, based on a novel hydrophilic surface chemistry. The LCP chip surface is saturated with single-stranded biotinylated DNA molecules, and liposomes tagged with cholesterol-labeled double-stranded DNA molecules are captured to the surface through DNA hybridization. For the details of reagents and techniques in this graph, refer to Bio-Rad bulletin 6161.

6.4 Experimental Design

How do I design an SPR experiment and what factors should be taken into consideration?

There are two major steps in an SPR experiment: ligand immobilization and analyte injection. All the factors affecting these two major steps, including the pre-steps and post-steps to enhance the performance of these two major steps, should be taken into consideration. The subsequent data processing and analysis should also be taken into consideration during experimental design. Please refer to Chapter 4 for details.

For an SPR experiment, how do I estimate the theoretical maximum analyte-ligand interaction response R_{max}?

First, measure the ligand immobilization response R_L from the ligand step. Second, use the equation below to calculate theoretical maximum analyte-ligand interaction response R_{max}.

$$R_{max} = n \frac{M_A}{M_L} R_L$$

n — stoichiometric number of the analyte-ligand interaction

M_A — analyte molecular weight

M_L — ligand molecular weight

For example, if an antibody (ligand) of 150 kD is immobilized to 1,000 RU, an antigen (analyte) is 30 kD, and the interaction ratio is 1:1. The R_{max} is calculated as follows.

$$R_{max} = 1 \times 30/150 \times 1{,}000 = 200 \text{ RU}$$

There are two channel referencing and two double referencing options. What do they mean and which should I use for my experiment?

Channel referencing is the minimum referencing required for SPR analysis. Double referencing is the secondary referencing that is used with the primary referencing in certain applications such as ligand-capture surfaces (Figure 6.5).

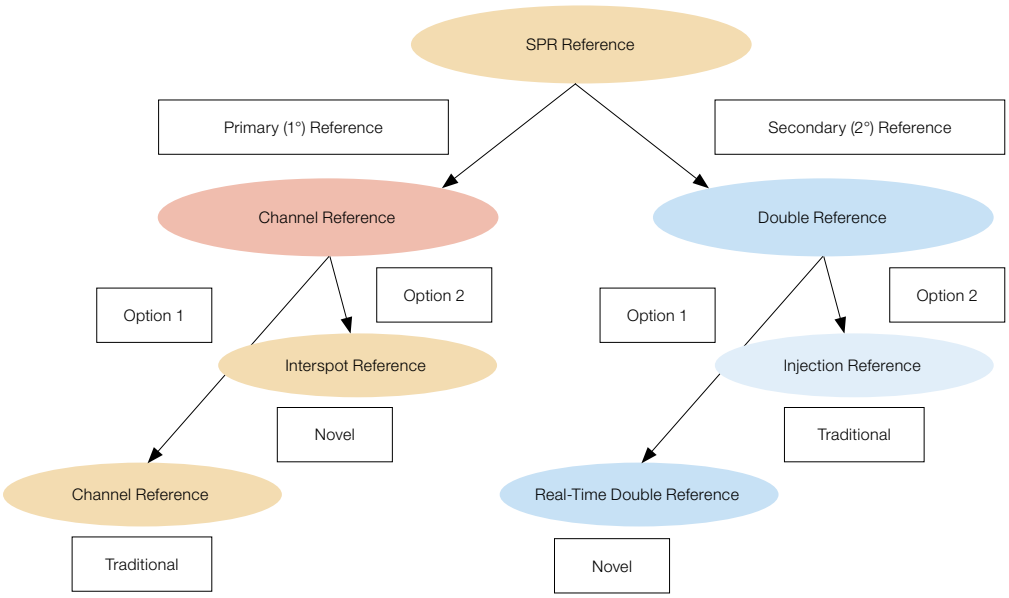


Fig. 6.5. SPR referencing options provided by the ProteOn XPR36 system.

The ProteOn XPR36 system offers two channel referencing options: interspot referencing, the novel referencing mechanism utilizing the blank surfaces between interaction spots, and channel referencing, the traditional SPR referencing mechanism that uses a dedicated blank channel. The innovative fluidics design of the ProteOn XPR36 system also offers two double referencing options: injection referencing, the traditional referencing mechanism that uses a blank running buffer injection prior to analyte injections, and real-time double referencing, a blank real-time running buffer injection performed in parallel with analyte injections. The ProteOn XPR36 system is the only SPR biosensor to feature real-time double referencing that runs simultaneously with the ligand-analyte interactions.

Interspot and real-time double referencing are unique to the ProteOn XPR36 system and their advantages are listed in Figure 6.6.

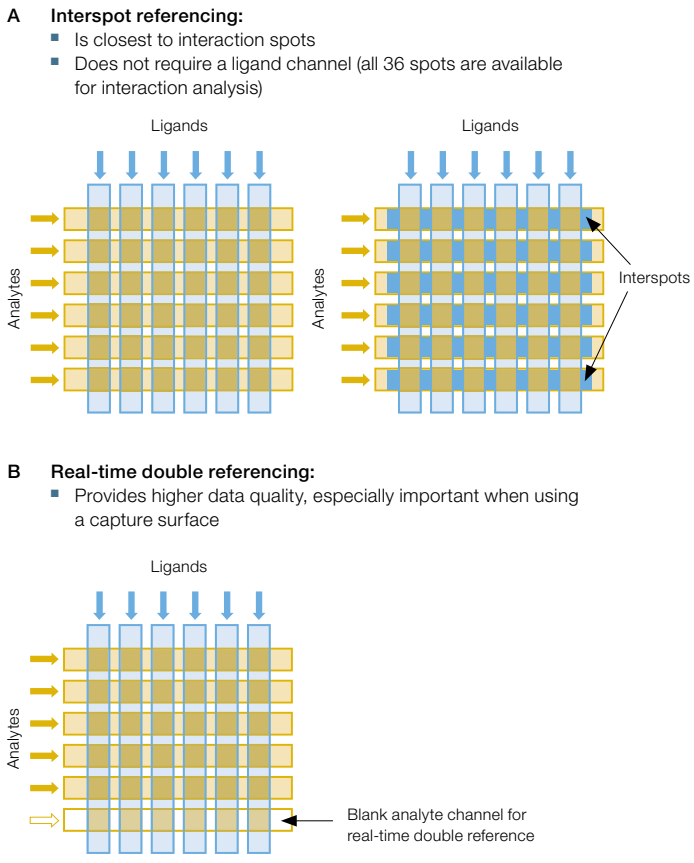


Fig. 6.6. The unique interspot referencing (A) and real-time double referencing (B) in the ProteOn XPR36 system. Saving interaction spots and providing high data quality.

What are the advantages of using a capture surface for ligand immobilization compared to direct amine coupling of the ligand?

The captured ligand has a better recovery yield because the capture mechanism results in the correct orientation of the ligand molecules. It is also easy to remove the ligand and generate a new ligand surface. Capture surfaces can be used for capturing ligands from crude samples, where amine coupling surfaces should not. Sometimes capture surfaces exhibit drift, or leaching, of the captured ligands from the chip surface. This can be completely resolved using the unique real-time double referencing in the ProteOn XPR36 system.

How do I correct the baseline drift when using a capture surface?

A capture surface uses a reagent to reversibly capture a ligand to the surface instead of covalently immobilizing the ligand. It has two main advantages: ease of surface regeneration and compatibility with non-purified ligand samples. Sometimes capture surfaces exhibit drift, or leaching, of the captured ligands from the chip surface. This can be completely resolved using the unique real-time double referencing in the ProteOn XPR36 system.

The solution to baseline drift is referencing the sensorgram to a blank running buffer injection (Figure 6.7). The ProteOn XPR36 system features unique real-time double referencing to correct this effect, providing the best referencing accuracy. The ProteOn XPR36 system is the only SPR biosensor to feature real-time double referencing that runs simultaneously with ligand-analyte interactions.

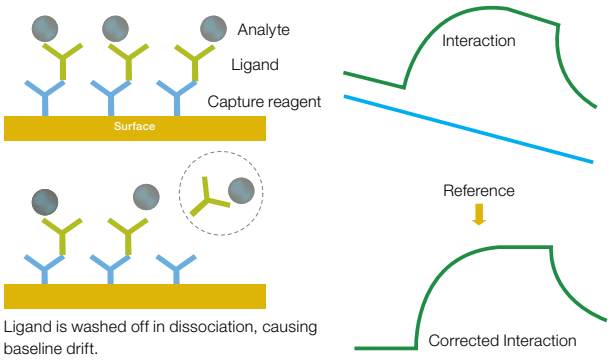


Fig. 6.7. The baseline drift, when using a capture surface, can be completely resolved using the real-time double referencing option in the ProteOn XPR36 system.

For robust kinetics, how many concentration points should be analyzed?

Kinetic analysis requires at least three dose-responsive sensorgrams. A single analyte injection on the ProteOn XPR36 system collects a set of six sensorgrams (five sensorgrams if the real-time double referencing is applied), that is one per analyte concentration. In the data analysis section of the software, the user may select the three to five best sensorgrams for analysis using the following criteria:

- Good sensorgram reproducibility
- An analyte concentration in the range of $0.1K_D$–$10K_D$

6.5 Experimental Tips

Should I degas the buffers and samples?

It is not required to degas the running buffer because the ProteOn XPR36 system has an inline degasser. However, samples should be degassed if they contain air bubbles.

How do I prevent evaporation of samples when working with microplates? Should I cover the plate with a simple seal?

Please use the sealing film that is provided with the ProteOn XPR36 system for microplates. Additional film can be ordered from Bio-Rad Laboratories.

I have experiment protocols from sensor chips coated with a carboxylated dextran layer. Can I apply them on ProteOn sensor chips that are coated with a carboxylated alginate layer?

Yes. In rare cases some protocols may need to be slightly adjusted due to the different surface chemistry.

The carboxylated dextran surface is a highly charged polymer, which may be difficult to activate and require high concentrations of activation reagents. This high-surface charge can cause nonspecific binding of some analytes.

The carboxylated alginate surface of ProteOn sensor chips is easily activated and has a low charge density. Compared to the carboxylated dextran surface, the carboxylated alginate surface usually requires fewer activation reagents.

Why does the ProteOn XPR36 system intentionally create separation air bubbles during sample uptake?

Separation air bubbles are intentionally created between the sample and the running buffer to prevent mixing. You may choose how many bubbles are generated based on the injection quality required.

Which sensor chips are compatible with running buffer containing free amine salts, such as Tris-HCl?

All sensor chips are compatible with running buffer with free amine salts. However, avoid using these salts when immobilizing a ligand by amine coupling.

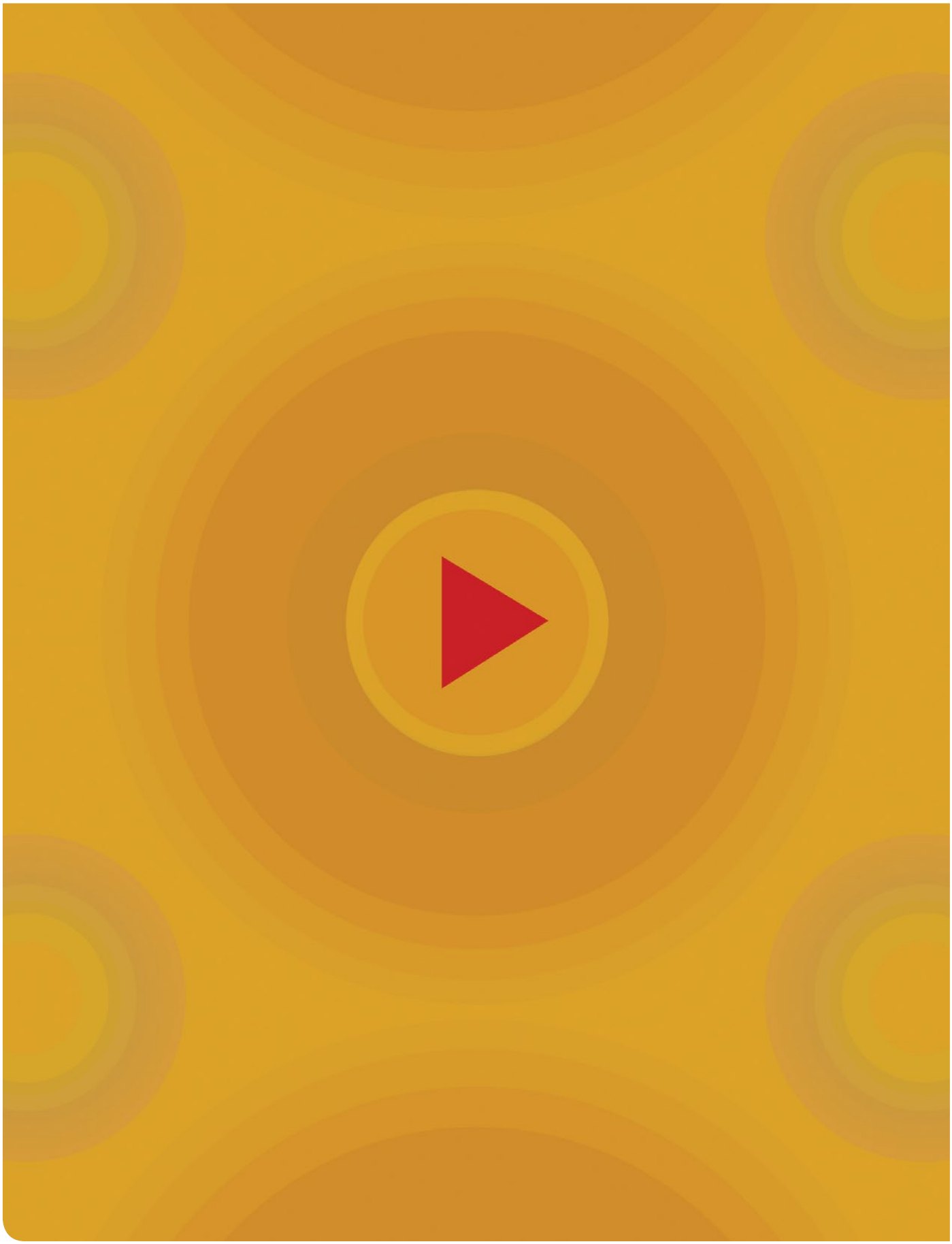
What is the mass transport effect and how can it be minimized?

The mass transport effect refers to the conditions where the transport rate or accessibility of the analyte to the ligand on the surface is restrained. Thus, the kinetic constant measurement is affected by the transport rate. This effect is typically due to fast on-rate and/or very high surface density of the ligand. Common solutions include reducing the ligand surface density and/or increasing the analyte injection flow rate. It should be noted that the mass transport effect is not a problem if its influence is insignificant to the data fitting. Normally in SPR experiments, biochemical factors such as ligand immobilization chemistry or analyte sample preparation have more influence on the accuracy of SPR analysis.

To quickly check for the mass transport effect: (1) Inject the analyte at different flow rates. If the same k_a is measured at all flow rates, there is no influence from the mass transport effect. But if the k_a decreases with decreasing flow rates, the system is mass transport limited. (2) Analyze data first with the Langmuir model and then with the Langmuir with mass transport model. If the same k_a values are obtained, there is no influence from the mass transport effect. If the k_a is lower with Langmuir analysis, then the system is mass transport limited.

When and why do I apply excluded volume correction?

Refer to Chapter 4, section 4.6. In experiments where analytes are dissolved in a cosolvent with a high refractive index, such as DMSO, the reference surface produces a larger bulk solvent response than the ligand surface because of the larger concentration of cosolvent near the reference surface. It is due to the exclusion of cosolvent by the ligand near the ligand surface. This difference in bulk effect causes inaccurate reference subtraction. Excluded volume correction uses a dilution series of DMSO solutions to correct for the difference of bulk effect between interaction and reference surfaces.



CHAPTER 7
Quick Guides

The quick guides outline the workflows for writing an experimental protocol and running an experiment on the ProteOn™ XPR36 system.

7.1 Writing a ProteOn XPR36 Experiment Protocol

Surface plasmon resonance (SPR) is a biosensor technology that measures biomolecular interactions in a real-time and label-free manner. The ProteOn XPR36 protein interaction array system is an SPR platform that utilizes the novel technology "XPR36" to enable parallel flow channels and crisscross microfluidics. It enables the creation of a 6 x 6 interaction array on a sensor chip.

This guide is written for the regular experiment layout in the ProteOn XPR36 system, which includes injecting ligands in vertical channels and analytes in horizontal channels.

A ProteOn Manager™ software protocol for an experiment consists of seven basic phases as shown below.

- Setting (optional)
- Conditioning
- Immobilization
- Stabilization
- EVC calibration (for applications with DMSO-containing running buffer)
- Interaction
- Regeneration (optional)

1. **Setting:** This phase ensures the system is ready to perform the experiment. It is used to set the chip temperature, flush the system with running buffer, and allow time for the system to come to thermal equilibrium after instrument startup.

Step Details

Step Type Set Temperature
Set Buffer

2. **Conditioning:** This phase prepares the chip surface for use. It is optional, but highly recommended because it increases baseline stability. The protocols of conditioning are listed as follows.

Note: For the LCP chip used in the ProteOn liposome capturing kit, conditioning is performed after the biotin-ssDNA capture step and before the liposome capture step.

Step Details

GLC, GLM, and GLH Chips

Step	Reagent	Orientation	Volume	Flow Rate
1	0.5% SDS	Horizontal	30 µl	30 µl/min
2	50 mM NaOH	Horizontal	30 µl	30 µl/min
3	100 mM HCl	Horizontal	30 µl	30 µl/min
4	0.5% SDS	Vertical	30 µl	30 µl/min
5	50 mM NaOH	Vertical	30 µl	30 µl/min
6	100 mM HCl	Vertical	30 µl	30 µl/min

NLC Chip

Step	Reagent	Orientation	Volume	Flow Rate
1	50 mM NaOH	Horizontal	30 µl	30 µl/min
2	1 M NaCl	Horizontal	30 µl	30 µl/min
3	50 mM NaOH	Vertical	30 µl	30 µl/min
4	1 M NaCl	Vertical	30 µl	30 µl/min

HTG and HTE Chips

Step	Reagent	Orientation	Volume	Flow Rate
1	0.5% SDS	Horizontal	30 µl	30 µl/min
2	50 mM NaOH	Horizontal	30 µl	30 µl/min
3	100 mM HCl	Horizontal	30 µl	30 µl/min
4	300 mM EDTA	Horizontal	100 µl	30 µl/min
5	0.5% SDS	Vertical	30 µl	30 µl/min
6	50 mM NaOH	Vertical	30 µl	30 µl/min
7	100 mM HCl	Vertical	30 µl	30 µl/min
8	300 mM EDTA	Vertical	100 µl	30 µl/min

LCP Chip (Used in ProteOn Liposome Capturing Kit)

Step	Reagent	Orientation	Volume	Flow Rate
1	1.3 µM biotin-ssDNA	Vertical	50 µl	30 µl/min
2	20 mM CHAPS	Vertical	150 µl	30 µl/min

Step Type Regenerate

Orientation Refer to the tables

3. **Immobilization:** This phase immobilizes a ligand to the chip surface through either direct covalent attachment or binding to a capture reagent. Consider the following factors before immobilizing ligands:

- Desired ligand immobilization level — determine the immobilization level (R_L), or amount of ligand immobilized on the chip surface, using the following equation:

$$R_{max} = n \frac{M_A}{M_L} R_L$$

R_{max} is the theoretical maximum response when the ligand interacts with an analyte, M_A is the molecular weight of the analyte, M_L is the molecular weight of the ligand, and n is the stoichiometric number of the interaction (analyte/ligand). For high-quality kinetic analysis, it is recommended to aim for analyte response with $R_{max} \leq 200$ RU.

The approximate capacity of the amine coupling chips (GLX: GLC, GLM, and GLH), the biotin capture chip (NLC chip), and the histidine-tag capture chips (HTX: HTG and HTE) are listed as follows:

GLC ~ 8 kRU GLM ~ 12 kRU GLH ~ 20 kRU
NLC ~ 2 kRU HTG ~ 5 kRU HTE ~ 12 kRU

- Optimize the immobilization conditions — in a ProteOn XPR36 system, immobilization conditions are optimized by injecting ligand across multiple channels with each channel containing the ligand at a different condition, such as concentrations or pH. Kinetic analysis is performed for all ligand channels at once.

- For amine coupling chips (GLC, GLM, and GLH chips) — typically, activation reagents for amine coupling chips are used at concentrations of 20 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 5 mM N-hydroxysulfosuccinimide (sulfo-NHS), but could be diluted in distilled water when a low immobilization level is needed. Activation reagents should be mixed immediately prior to injection. Contact time is 60 sec for moderate activation and 300 sec for high activation levels. Usually the ligand is prepared in the concentration range of 0.5–25 µg/ml. Ligand coupling buffer is typically 1 pH unit lower than the ligand pI to facilitate charge attraction between the positive ligand and the negative chip surface. Low ionic strength is also required to enhance the charge attraction.

Note: It is possible to use the co-inject function in the ProteOn XPR36 system to remove the manual mixing of the activation reagents. Refer to the article "Ligand Immobilization in Protein Interaction Studies — An Unattended Amine Coupling Protocol with Automatic Coinjection Activation" at bioradiations.com (May 16. 2012).

Step Details

Step Type: EDC/sulfo-NHS (for amine coupling chip) or $NiSO_4$ (for HTG and HTE chips) – Activate

Ligand – Ligand

Ethanolamine (for amine coupling chip) or biotin (for NLC and LCP chips) – Deactivate

Orientation: Vertical

Note: NLC and LCP chips do not need activation, and deactivation with biotin is optional. HTG and HTE chips need activation but not deactivation.

4. Stabilization: This phase removes unattached ligand from the chip surface and stabilizes the baseline after multichannel module (MCM) rotation. Allowing the baseline to stablize for 30 min is recommended to obtain high-quality kinetic analysis. The phase includes performing one or more injections, which may consist of either or both of the following. For NLC chip, injection of 1 M NaCl following the running buffer is recommended.

✓ Running buffer

✓ Any regeneration solution compatible with the ligand

Step Details

Step Type: Running buffer – Blank
Regeneration solution – Regenerate

Orientation: Horizontal

5. EVC Calibration: This phase accounts for excluded volume effect. Follow Chapter 4, section 4.6 for setting up the steps. Calibration is needed when DMSO is used as cosolvent in sample and running buffer in small molecule application.

Step Details

Step Type: EVC Calibration

Orientation: Horizontal

6. Interaction: This phase analyzes the interaction between the ligand and the analyte. The following factors should be considered.

1. Prepare the analyte samples in the running buffer to form a dilution series, typically a two- or threefold dilution series centered at the expected K_D value.
2. When needed, set up a double reference. Replace one of the six analyte channels with running buffer for use as a real-time double reference (row reference). Alternatively, set up an injection of running buffer to all six analyte channels prior to the injection of analyte samples (injection reference). Double referencing is needed to correct for baseline drift, and is used mostly when the ligand is captured reversibly by a capture reagent, such as antibody, NeutrAvidin (NLC and LCP chips), or tris-NTA complex (HTG and HTE chips).
3. Ensure that the contact time of the interaction is long enough to observe curvature in the association phase.
4. Ensure that the dissociation time of the interaction is long enough to observe adequate signal drop in the dissociation phase.
5. Optimize flow rate to reduce the mass transport effect while minimizing sample consumption.

Step Details

Step Type: Injection reference – Blank
Analyte – Analyte

Orientation: Horizontal

Note: The injection parameters of analyte samples may be customized, and those of the injection reference should be the same for correct referencing.

7. Regeneration: This phase regenerates the chip surface with ligand or capture reagent.

Step Details

Step Type: Regenerate

Orientation: Horizontal

Note: Conditions should be optimized for each interaction. Regeneration should remove all the bound analyte but not damage the ligand.

7.2 Running an Experiment with the ProteOn XPR36 System

7.2.1 Instrument Preparation

1. Start the instrument. (Skip this step if the instrument is in continuous use.)

A. Press the power button on the left side of the instrument to the 1 position to turn on the instrument.

B. Turn on the user computer.

C. Wait until all five instrument LEDs turn yellow.

D. Launch ProteOn Manager™ software.

E. Wait until all five instrument LEDs and **Communication state** in the software turn green.

F. If the instrument is started after long-term shutdown, follow the pop-up instruction in the software to flush the fluidic system.

2. Initialize the sensor chip.

Note: If the sensor chip to be used is already in the instrument, start from step D if it is not initialized. Press **Resume** if the Instrument State is in Standby.

A. In the Navigator panel, go to the Instrument tab and select **Instrument Control**.

B. If there is a sensor chip in the instrument, press **Eject** to eject it.

C. Insert the sensor chip to be used. Wait until the Initialization Status box shows Chip Not Initialized.

D. Select the chip initialization method and press **Initialize Chip**. For glycerol initialization, follow the pop-up instruction to place the normalization solution in the instrument.

E. Wait until the Initialization Status box shows Chip Initialized (Type), indicating that the chip initialization is completed.

7.2.2 Running an Experiment

1. Write a protocol.

Note: Refer to section 7.1.

A. In the menu bar, click **File** and select **New** to open the database browser. Select **New Protocol** and press the **New Protocol** button to start with a blank protocol, or select an existing protocol and press **New Protocol** to copy the selected protocol.

Note: In the database browser, Protocol means the set of instrument parameters, sample information, and running steps for an experiment. Template means a saved protocol intended for reuse, and Experiment means an implemented protocol. You are able to create a new protocol from all these file types.

B. The software will automatically go to the Protocol tab. Select **Configuration**. Edit the protocol name, sample container, and other information as needed.

C. Select **Samples** and input the information of all the samples and reagents in the corresponding sample wells.

D. Select **Steps**. In the Protocol Editor panel, drag steps one by one from the left side. In the Step Details panel, click the arrow in the sample source table and select the correct sample. If needed, adjust the step setting such as Flow Rate, Contact Time, and Volume.

E. Select **Protocol Check** and review the protocol steps.

F. If a printed copy of the protocol or the sample layout is needed for sample preparation, select **Protocol Report** or **Sample Report**.

2. Run a protocol.

A. Prepare and load the samples in the sample container. The sample container must be either a rack or microplates and consistent with the sample container information in the Configuration screen. Place the sample container in the instrument correctly positioned with sample vial/well A1 at bottom-left corner.

B. In the Navigator panel, go to the Run tab and select the protocol in the Selected Protocol/Experiment box.

C. Click **Run** to start the experiment.

Note: If there is any non-timeout pause step in the protocol, you will have to click **Run** in that step to continue the experiment.

7.2.3 Instrument Maintenance

1. Run post-experiment maintenance. (Skip this step if the sensor chip will be reused immediately.)

A. In the Navigator panel, go to the Instrument tab and select **Instrument Control**. Click **Eject** to eject the sensor chip.

B. Insert an MNT chip. Wait until the Initialization Status box shows Maintenance Chip. The software will automatically choose the Maintenance screen.

C. Click **Post-Experiment**. Follow the pop-up instruction to load the reagents and click **Next** to start the maintenance protocol.

Note: The ProteOn Manager software also indicates in the Maintenance Status table whether other maintenance protocols are required at this time.

2. Shut down the instrument. (Skip this step if the instrument is in continuous use.)

A. In the Navigator panel, go to the Instrument tab and select **Instrument Control**.

B. Click **Shutdown** and select either **Immediate Shutdown** or **Long Term Shutdown**. An MNT chip in the instrument is required for long-term shutdown. Follow the pop-up instruction to load the reagents, and click **Next** to start the shutdown process.

C. Wait until the shutdown process is completed. Press the power button on the left side of the instrument to the 0 position to turn off the instrument.

Note: If the instrument is in continuous use, it is recommended to keep the system in distilled water during the idle time; for example, overnight or over the weekend.

7.2.4 Import/Export Experiment Files

1. Export experiment files.

A. In the menu bar, click **File**, select **Export**, and choose **Experiment/Protocol File** in the submenu to open the database browser. Select the experiment file to export. Hold the **Ctrl** key to select multiple experiment files.

B. Click **Export** and select the target folder to store the exported experiment files.

C. Click **Save** to start the export process. When completed, click **Close**. The exported file has the extension name “pomexp” and it can be imported only to ProteOn Manager software.

Note: It is possible to export the data in an experiment file in a spreadsheet format. Please refer to the user manual for details.

2. Import experiment files.

A. In the menu bar, click **File**, select **Import**, and choose **Experiment/Protocol File** in the submenu to open the file browser. Select the experiment file to import. Hold the **Ctrl** key to select multiple experiment files.

B. Click **Open** to start the import process. When the import process is completed, click **Close** to close the database browser. The imported experiment file is added to the database.

Note: If the Import and Export options are grayed out, check that the ProteOn Manager USB key is in place.

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