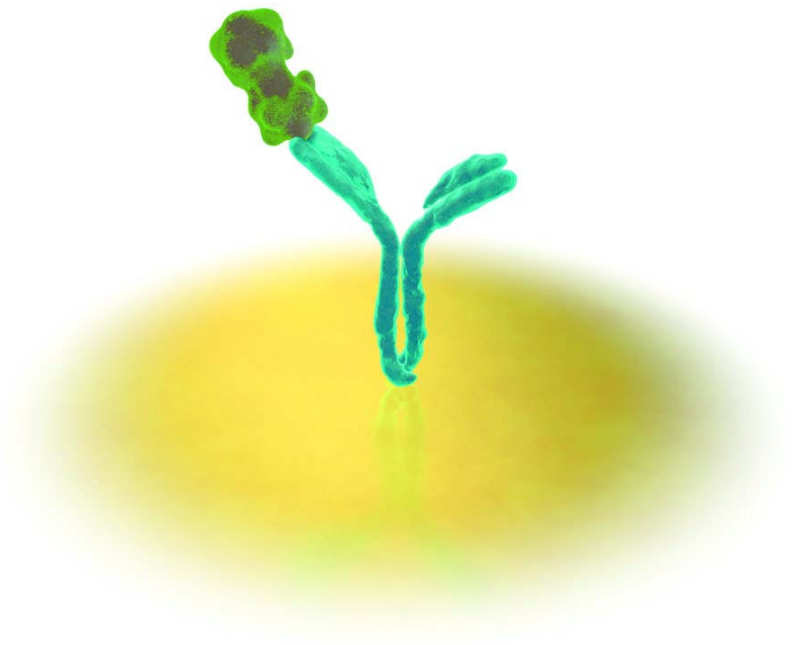
GE Healthcare
Life Sciences

Biacore™ Assay Handbook



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1 Introduction

1.1 What Biacore™ systems measure

Biacore systems monitor molecular interactions in real time, using a non-invasive label-free technology that responds to changes in the concentration of molecules at a sensor surface as molecules bind to or dissociate from the surface. The detection principle is based on surface plasmon resonance (SPR), that is sensitive to changes in refractive index within about 150 nm from the sensor surface. To study the interaction between two binding partners, one partner is attached to the surface and the other is passed over the surface in a continuous flow of sample solution. The SPR response is directly proportional to the change in mass concentration close to the surface.

Biacore systems can be used to study interactions involving (in principle) any kind of molecule, from organic drug candidates to proteins, nucleic acids, glycoproteins and even viruses and whole cells. Since the response is a measure of the change in mass concentration, the response per molar unit of interactant is proportional to the molecular weight (smaller molecules give lower molar responses). The practical lower limit for detection of small molecules with today's instrumentation is about 100 Da.

The detection principle does not require any of the interactants to be labeled, and measurements can be performed on complex mixtures such as cell culture supernatants or cell extracts as well as purified interactants. The identity of the interactant monitored in a complex sample matrix is determined by the interaction specificity of the partner attached to the surface. The SPR detection principle is non-invasive and works equally well on clear and colored or opaque samples.

1.2 Where Biacore systems are used

Measurements with Biacore systems provide valuable information in a number of application areas:

- detection of specific molecules such as anti-drug antibodies in immunogenicity studies
- screening for binding partners and ranking of binding ability, for instance in drug discovery and biopharmaceutical development
- determination of simultaneous interaction capabilities, for example epitope mapping with monoclonal antibodies

- determination of interactant concentration in samples, from measurements under conditions where interaction levels can be related to concentration
- measurement of interaction kinetics and affinity, made possible by real-time detection of interaction events

Biacore systems are used primarily in pharmaceutical development, quality control and basic life science research.

1.3 Scope of this book

This book gives a general overview of the different types of Biacore-based applications, together with advice and recommendations on preparation of the sensor surface, preparation of samples and design and optimization of different assays. Troubleshooting guidelines to help in interpreting unsatisfactory results are also included.

The information is presented in general terms, without reference to specific Biacore systems. Use this handbook together with the specific user documentation for your Biacore system, which will describe how to implement the assay principles in the instrument and system software.

1.4 Biacore system range

All Biacore systems exploit the same detection principle and use essentially the same range of sensor surfaces (see Section 3.1), with a controlled flow system for delivery of samples and reagents to the sensor surface. Individual systems differ primarily in capacity and degree of automation, and are suited to use in different laboratory contexts:

- Entry-level systems like Biacore X100 have one or two pairs of flow cells (where one cell in a pair is used for detecting the interaction and the other as a reference cell where required) and can handle only relatively few samples without user intervention.
- Advanced systems may have multiple detection spots (in Biacore 4000, for example, 20 detection spots are divided between 4 flow cells for parallel analysis of multiple samples) and unattended sample handling capacity for 1500 samples or more.

While the available options for assay development and execution may vary between the different systems, the same general principles apply to all systems. This book will focus as far as possible on common principles of Biacore assays, keeping system-specific information to a minimum.

1.5 Biacore terminology

Biacore systems monitor the interaction between two molecules, of which one is attached to the sensor surface and the other is free in solution. The following terms are used in the context of Biacore-based assays:

- The interaction partner attached to the surface is called the *ligand* (Figure 1-1). In drug discovery and development work, the ligand is sometimes referred to as the *target molecule*.

  Note: *The term "ligand" is applied here in analogy with terminology used in affinity chromatography contexts, and does not imply that the surface-attached molecule is a ligand for a cellular receptor.*

- The ligand may be attached to the surface either by covalent *immobilization* using chemical coupling reagents or by capturing through high affinity binding to an immobilized *capturing molecule*.
- The *analyte* is the interaction partner that is passed in solution over the ligand (Figure 1-1).

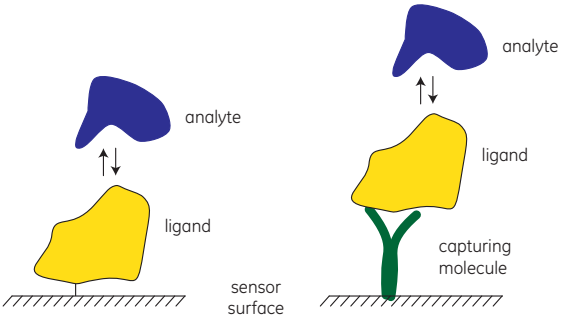


Figure 1-1. The ligand is the interaction partner that is attached to the sensor surface. The ligand may be immobilized directly on the surface (left) or attached through binding to an immobilized capturing molecule (right). The analyte is free in solution and binds to the immobilized ligand.

- In indirect assay formats the analyte is detected using a secondary *detecting molecule*, which can bind to both analyte in solution and ligand on the sensor surface. The observed response is derived from binding of detecting molecule to the ligand: the presence of analyte in the sample inhibits this binding so that the response is inversely related to the amount of analyte.
- Analysis is performed by injecting sample over the surface in a carefully controlled fashion. The sample is carried in a continuous flow of buffer, termed *running buffer*.
- Response is measured in *resonance units* (RU). The response is directly proportional to the concentration of biomolecules on the surface.

- A *sensorgram* is a plot of response against time, showing the progress of the interaction (Figure 1-2). This curve is displayed directly on the computer screen during the course of an analysis.
- A *report point* records the response on a sensorgram at a specific time averaged over a short time window, as well as the slope of the sensorgram over the window. The response may be absolute (above a fixed zero level determined by the detector) or relative to the response at another specified report point (Figure 1-2).

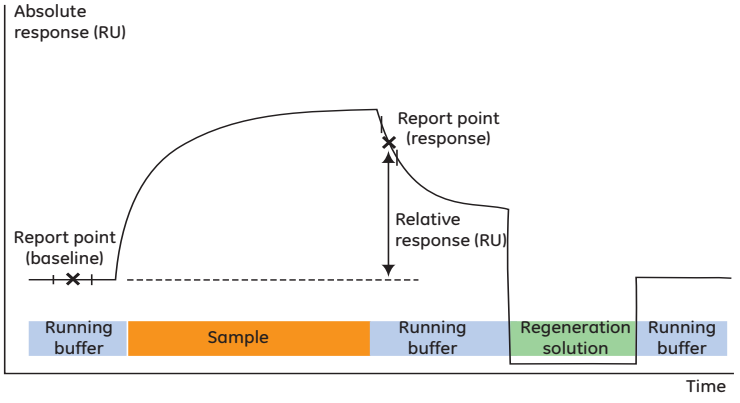


Figure 1-2. Schematic illustration of a sensorgram. The bars below the sensorgram curve indicate the solutions that pass over the sensor surface.

- *Regeneration* is the process of removing bound analyte from the surface after an analysis cycle without damaging the ligand, in preparation for a new cycle. For ligand that is captured rather than immobilized, regeneration usually removes the ligand and leaves the capturing molecule intact.
- SPR detection monitors changes in refractive index close to the surface, and differences in refractive index between running buffer and injected sample will be recorded as a rapid shift in response at the beginning and end of the injection. This is referred to as a *bulk refractive index effect* or *bulk shift*.
- Naming conventions for kinetic and affinity constants vary considerably in the literature. Throughout this book (and in other Biacore documentation), rate constants are identified with lower-case letters (k_a for association rate constant and k_d for dissociation rate constant), while equilibrium or affinity constants are written with upper-case letters (K_A for the equilibrium association constant and K_D for the equilibrium dissociation constant).

2 Application overview

This chapter gives an overview of the major application areas for Biacore systems. These are

- Screening for binding partners, including immunogenicity testing
- Measurement of interaction kinetics and affinity
- Measuring concentration
- Mapping binding site topography

Subsequent chapters in this Handbook consider screening, kinetics/affinity and binding site topography in more detail. Measurement of concentration is discussed in a separate Handbook (*Biacore Concentration Analysis Handbook*).

2.1 Screening and detecting binding partners

Because Biacore systems exploit surface plasmon resonance (SPR), a generic detection technology that requires no labels, they are well suited to screening for binding partners in pharmaceutical and biopharmaceutical development and immunogenicity testing.

The response in a Biacore system is directly related to the change in mass concentration on the surface, so that molar responses (i.e. responses for a given number of molecules) are proportional to the size of the molecule involved. A given response will represent a higher molar concentration of a small molecule than a large one: conversely, a given number of molecules binding to the surface will give a lower response if the molecule is small. The relationship between response and surface concentration is essentially constant for proteins regardless of amino acid composition and sequence, and is similar for most other biological macromolecules.

Note: *In early literature related to Biacore, 1 RU was stated to be approximately equivalent to a change in surface concentration of 1 pg/mm^2. This relationship holds roughly for proteins on Sensor Chip CM5. Beware of applying the conversion to non-protein molecules and other types of sensor chip. Response values from Biacore systems should always be quoted in RU, not in units of surface concentration.*

Measurements can be made on complex samples such as cell culture medium or body fluids: the specificity of the observed response is determined primarily by the choice of ligand attached to the surface. This is particularly useful in applications such as antibody screening and immunogenicity testing: for example, screening with an antigen as ligand will detect only specific antibodies, while using an ligand directed towards a common region of a given antibody class will detect that class regardless of antigen specificity.

2.2 Kinetics and affinity measurements

Determination of interaction kinetics is perhaps the most characteristic application for Biacore systems. The label-free real-time detection allows interactions to be monitored with high resolution as they happen, and the results can be interpreted in relation to a mathematical model of the interaction mechanism to evaluate kinetic parameters (association and dissociation rate constants). Affinity constants, which reflect the strength of binding but not the rate, can be derived either from the rate constants or from analysis of the level of binding at steady state. The theory of kinetic and affinity analysis is described in Appendix A.

Note: *The term "kinetics" is used in this handbook to refer to interaction kinetics. The kinetics of other processes such as enzyme-catalyzed reactions are not normally amenable to study in Biacore systems.*

2.2.1 Kinetic analysis

Interaction kinetics are analyzed by monitoring the interaction as a function of time over a range of analyte concentrations, and then fitting the whole data set to a mathematical model describing the interaction. The association phase (during sample injection) contains information on both association and dissociation processes, while only dissociation occurs during the dissociation phase (after sample injection, when buffer flow removes dissociated analyte molecules).

It is important to be aware that the results of kinetic analysis are relevant only in the context of the interaction model chosen for the evaluation. It is not strictly possible to derive a model from the observed binding behavior, although the shape of the sensorgrams can sometimes give clues for the choice of appropriate models. Fitting the data to a mathematical model does not provide evidence of the interaction mechanism (Figure 2-1).

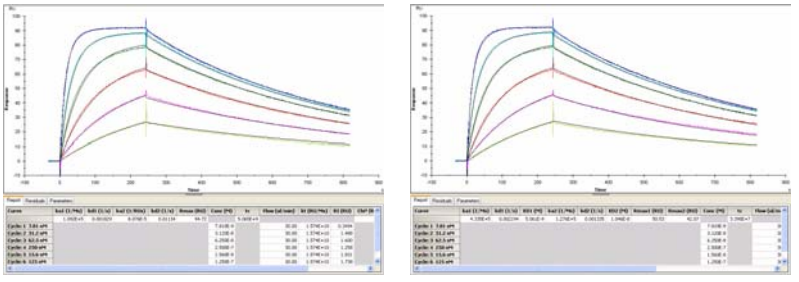


Figure 2-1. Fitting experimental data to a mathematical model does not prove that the model is appropriate, and the same data may fit acceptably to several models. In this example, the closeness of fit cannot distinguish confidently between a bivalent analyte model (left) and a heterogeneous ligand model (right).

Assay set-up

There are currently two ways of setting up a kinetic analysis experiment (Figure 2-2):

- Multi-cycle kinetics runs each analyte concentration in a separate cycle, regenerating the surface after each sample injection. It is important that regeneration is optimized (see Biacore Sensor Surface Handbook for more information) so that the surface properties are consistent from cycle to cycle.
- Single-cycle kinetics runs a series of analyte concentrations in one cycle, with no regeneration between sample injections. This approach is valuable in situations where acceptable regeneration cannot be achieved, but is more sensitive to response drift since the cycle time is longer.

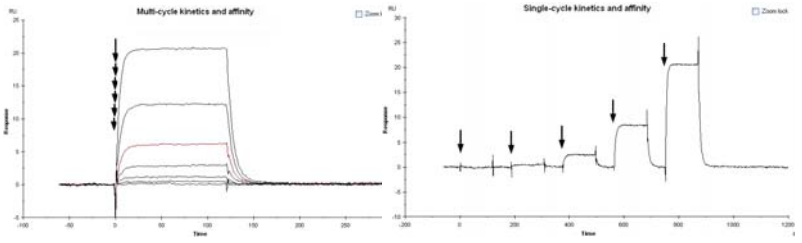


Figure 2-2. In multi-cycle kinetics and affinity determinations (left), each sample is injected in a separate cycle. The concentration series is presented as an overlay plot aligned at the start of the injection in the evaluation software.
In single-cycle determinations (right), the samples are injected sequentially in the same cycle.
Arrows in the illustrations mark the start of sample injections.

2.2.2 Affinity analysis

Interaction affinity may be determined in three independent ways using Biacore systems: calculation from kinetic constants, measurement of steady-state binding levels and determination of affinity in solution.

Affinity constants from kinetics

For simple 1:1 binding, the affinity constant is equal to the ratio of the rate constants (equilibrium association constant $K_A = k_a/k_d$, equilibrium dissociation constant $K_D = k_d/k_a$, see Appendix A). Values for affinity constants can therefore be derived from kinetic measurements.

Note that these values, like the values for rate constants, are only valid in the context of the model used to analyze the binding data. More complex binding mechanisms do not always give straightforward relationships between affinity and kinetics, and it may only be possible to derive affinity constants from kinetic constants for values obtained with a simple interaction model.

Steady state affinity

Analyzing the level of steady state or equilibrium binding as a function of interactant concentrations is the foundation for many standard techniques for determining interaction affinity. The basic relationship between concentrations of interactants and complex and the affinity is described in Appendix A. Affinity constants may be derived from the experimental data from linearized plots such as Scatchard plots or (as supported in Biacore software) by direct analysis of a plot of amount of complex against analyte concentration (Figure 2-3).

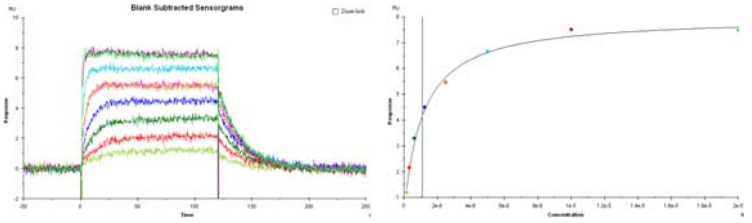


Figure 2-3. Sensorgrams (left) and corresponding plot of steady state response against concentration (right) for determination of binding affinity. The vertical line in the right-hand plot indicates the value of the calculated equilibrium dissociation constant K_D.

Analysis of steady-state binding data by fitting the curve of binding level against concentration can only be applied to 1:1 binding. A model for 1:1 binding at two independent ligand sites can be useful in some circumstances, but more complex models are not sufficiently robust for analysis of the data. It is however possible to apply classical Scatchard analysis to data obtained from Biacore systems, to give some indication of the causes of deviation from 1:1 binding behavior.

It may be thought that the same experiment can in principle provide data for both kinetics and steady state binding, offering two independent affinity values from one set of data. In practice, however, restrictions on the injected volume of sample (and therefore the contact time between sample and the sensor surface) mean that interactions that give measurable kinetics seldom reach steady state and *vice versa*. The affinity for a given interaction can therefore usually be derived from kinetics or steady state measurements but seldom both.

Affinity in solution

The principle of measuring affinity in solution with Biacore systems is to determine the free concentration (and therefore the concentration of complex) in mixtures of known interactant concentrations, using the Biacore system for the concentration assay. Commonly, the assay is set up with one interactant A (or an analogue thereof) on the surface. A calibration curve is established for determining the concentration of the second interactant B. Mixtures containing variable concentrations of A and a fixed concentration of B are allowed to reach equilibrium and then analyzed for the free concentration of B. The results are analyzed using standard tools for affinity measurements. A requirement of this

approach is that only free B and not complex AB can bind to the surface-attached A.

While the requirement for calibrated concentration measurements of analyte B makes this approach somewhat cumbersome, the main advantage is that samples can be incubated as long as necessary to reach steady state. Measurements are therefore not limited by the available contact time between sample and sensor surface.

2.3 Concentration measurements

Biacore systems can be used for measurement of analyte concentrations because the amount of analyte that binds to the surface under appropriate conditions can be related to the concentration of analyte in the sample. In *calibrated assays*, the response is related to the analyte concentration through a calibration curve, prepared with standards containing known analyte concentrations. This approach is analogous to many other established methods of concentration measurement. *Calibration-free concentration analysis* (CFCA) is an alternative approach supported in some Biacore systems, where concentrations are derived from the observed rate of diffusion-limited binding without reference to a calibration curve.

Concentration assays using Biacore systems are described in detail in the Biacore Concentration Analysis Handbook.

2.3.1 Calibrated assays

Calibrated concentration measurements may be designed as *direct* or *indirect* assays.

- *Direct assays* measure analyte bound directly to the ligand on the sensor surface. This approach is suitable for macromolecular analytes (molecular weight > 5000 daltons): direct detection of smaller molecules is possible but the useful range of the assay is generally limited in such cases. *Response enhancement* or *sandwich* approaches can be used to amplify the response obtained and/or to increase the selectivity of the assay.
- *Indirect* or *competition assays* provide an indirect measure of analyte concentration, and are most useful for low molecular weight analytes. The commonest format for indirect assays is the *solution competition* or *inhibition* approach, where a known amount of an interacting partner called the *detecting molecule* is mixed with the sample, and the amount of free detecting molecule remaining in the mixture is measured. In the *surface competition* method, analyte and a high molecular weight analogue (often a protein conjugate) compete for binding to a common partner on the sensor chip surface. In both competition assay formats, the response obtained is inversely related to the concentration of analyte in the sample.

Measuring concentration in a calibrated assay, whether direct or indirect, relies on the ability to establish a calibration curve using standards of known concentration. The results for unknown samples are always obtained with reference to the standard, and represent "true" concentrations only insofar as the standard concentrations are reliable. More detailed discussion may be found in the Biacore Concentration Analysis Handbook.

2.3.2 Calibration-free concentration analysis (CFCA)

Calibration-free assays are based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration. The concentration is calculated from knowledge of the diffusion coefficient of the analyte together with analysis of the observed binding rate under partially diffusion-limited conditions. This approach can be useful in situations where no satisfactory calibrant is available for the analyte under study, or when it is important to determine "true" concentrations as closely as possible. Calibration-free assays are always set up in the direct binding format.

The requirement for binding under partially diffusion-controlled conditions limits the dynamic range of CFCA and restricts the approach to analysis of macromolecules (molecular weight above 5,000 Da).

2.4 Mapping binding sites

Binding sites on a macromolecule can be mapped by testing the ability of multiple binding partners to bind independently or to compete with each other. Two binding partners that are directed towards distinct binding sites will be able to bind simultaneously, while partners that are directed towards interfering sites will compete with each other. This kind of analysis is most common in studies of antibody specificity (where it is called *epitope mapping*, see Figure 2-4), although it can in principle be applied to any investigation of multiple binding sites on a macromolecule.

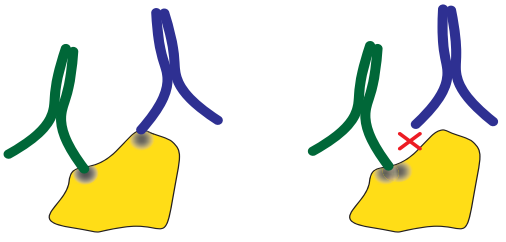


Figure 2-4. Different antibodies can bind simultaneously to distinct and spatially separated epitopes (left), but not to common or overlapping epitopes (right).

Epitope mapping can be performed using two distinct approaches:

- Pair-wise binding tests the ability of pairs of antibodies to bind simultaneously to the same antigen. Simultaneous binding is a clear indication of distinct epitopes, while interference can result from binding to the same or closely situated epitopes, or for example from a conformational change in the antigen induced by binding of one antibody that hides the epitope for the other.
- Peptide inhibition studies test the ability of peptides that represent specific regions on the antigen to inhibit binding of single antibodies. A peptide that blocks antibody binding is taken to represent at least a part of the physical epitope for that antibody.

3 General considerations

This chapter discusses aspects of working with Biacore systems that are common to all application areas.

3.1 Sensor surfaces

3.1.1 General properties

The sensor chip in Biacore systems consists of a glass slide coated with a thin layer of gold. These components, together with the docking system for mounting the sensor chip in the optical system, are required for generation of an SPR signal. To provide a suitable environment for the molecular interactions being studied, the gold surface is covered by a linker layer and (on most sensor chip types) a matrix of modified dextran (Figure 3-1). It is the surface matrix that determines the properties of the sensor chip with respect to ligand attachment and molecular interaction.

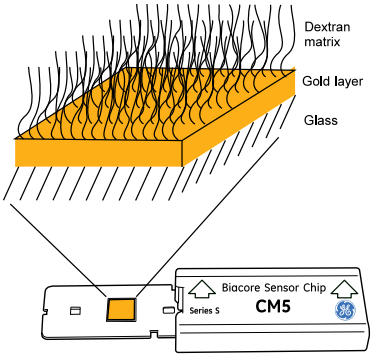


Figure 3-1. Schematic illustration of the structure of the sensor chip surface on CM-series chips.

3.1.2 Sensor surface types

GE Healthcare offers a range of sensor surfaces designed for different application requirements, as summarized in Table 3-1. Detailed descriptions of sensor chip types may be found in the Biacore Sensor Surface Handbook and on www.gelifesciences.com/biacore.

Table 3-1. Characteristics and application areas for sensor chips.

Sensor Chip	Characteristics	Use
CM-series sensor chips (carboxymethyl-dextran surface matrix)		
CM5	Moderate capacity	General purpose
CM7	High capacity	High immobilization levels for work with low molecular weight analytes (not suitable for protein analytes or capture applications).
CM4	Low capacity (low substitution density)	Low immobilization levels for kinetics. May help to reduce non-specific binding from complex samples.
CM3	Low capacity (shorter dextran chains)	Large particulate analytes (viruses and cells). Low immobilization levels for kinetics. May help to reduce non-specific binding from complex samples.
C1	Flat surface, no dextran matrix. Carboxyl groups directly attached to the surface linker layer. Restricted mobility of attached ligand.	Large particulate analytes (viruses and cells).
Hydrophobic surfaces		
HPA	No surface matrix or attached groups	Direct adsorption of lipid monolayers, with or without associated proteins.
L1	Dextran matrix substituted with lipophilic residues	Capture of intact lipid vesicles.

Sensor Chip	Characteristics	Use
Surfaces for ligand capture		
SA	Streptavidin covalently attached to dextran matrix	Permanent capture of biotinylated molecules
CAP	Oligonucleotide covalently attached to dextran matrix	Reversible capture of biotinylated ligands, mediated by a streptavidin-carrying complementary oligonucleotide.
NTA	Nitrilotriacetic acid covalently attached to dextran matrix	Capture of histidine-tagged ligands

3.2 Attaching the ligand

In broad terms, there are two main approaches to attaching macromolecular ligands to the sensor surface, covalent immobilization and high affinity capture. Details of these alternatives may be found in the Biacore Sensor Surface Handbook.

3.2.1 Covalent immobilization

Covalent immobilization involves irreversible chemical attachment of the ligand to the surface, usually to the carboxymethyl groups on CM-series sensor chips. The ligand remains on the surface throughout the lifetime of the sensor chip, and is regenerated to remove non-covalently bound molecules after each analysis cycle.

Covalent immobilization may exploit amine, thiol (either native or introduced) or aldehyde groups (obtained by oxidation of *cis*-diols) on the ligand. Amine coupling is the most widely used alternative.

Conditions for immobilization

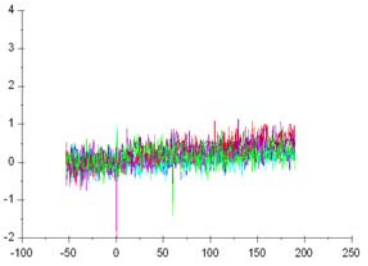
The concentration of ligand in the surface matrix for analysis in Biacore systems is many times higher than that in a typical bulk solution: immobilization of a protein at a response level of 100 RU on Sensor Chip CM5 corresponds roughly to a bulk concentration of 1 mg/ml in the surface matrix, while typical bulk concentrations used for immobilization are 50-100 µg/ml. In most immobilization methods, ligand is concentrated on the surface by a process of *electrostatic pre-concentration*. This occurs when the pH of the ligand solution is below the isoelectric point of the ligand, so that the ligand carries a net positive charge, but above the pKa (3.5) of carboxyl groups on the surface so that the

surface is negatively charged. Low ionic strength favors the electrostatic interaction, and buffers with 10-20 mM total cation concentration are generally recommended.

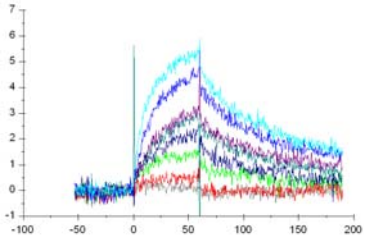
Choice of immobilization chemistry

Many macromolecules can be immobilized as ligands on the sensor surface without significantly interfering with the interaction being studied, provided that the coupling chemistry involves groups that are distant from the site of interaction. Chemistries such as amine coupling that target common groups may result in a mixture of ligand molecules on the surface with different points of attachment: in some cases, ligand may be partially inactivated with respect to interaction by the attachment, but this does not matter as long as sufficient active molecules are present. More specific chemistry such as thiol coupling may be used to reduce the variation in orientation of the attached molecules, or even to determine precisely how the molecules will be attached (if the ligand contains only a single target group for attachment).

Amine coupling
Immobilized: 10,000 RU
Activity: undetectable



Thiol coupling
Immobilized: 12,000 RU
Activity: 5%



Poly-histidine tag capture
Immobilized: 2,000 RU
Activity: 20%

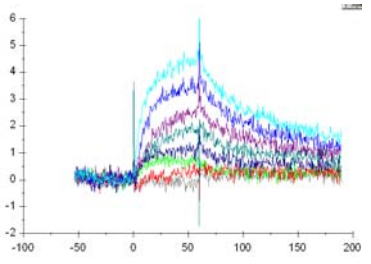


Figure 3-2. Amine coupling is frequently successful, but in this example (immobilization of a hormone receptor) amine coupling destroys the ligand activity completely. The best results are obtained with high affinity capture.

Amine coupling is generally applicable to a wide range of protein ligands, and is often the most convenient method of attachment. However, testing a range of attachment chemistries can be worthwhile in critical cases: Figure 3-2 illustrates results from immobilization of a hormone receptor where amine coupling gave a fully acceptable immobilization level but activity was preserved better by thiol coupling. High affinity capture of poly-histidine tagged receptor on Sensor Chip NTA gave however the best yield in terms of binding activity (tested by binding of hormone) per immobilized RU.

3.2.2 High affinity capture

High affinity capture refers to attachment of ligand by binding to an immobilized capturing molecule, and offers advantages over covalent immobilization in a number of situations:

- Capture does not require any chemical modification of the ligand and can be performed in physiological buffer conditions. It can therefore be used with ligands that are not amenable to covalent immobilization.
- Normally, regeneration of the surface with a captured ligand involves removal of ligand together with other bound molecules, leaving the capturing molecule ready bind fresh ligand for the next cycle. While this procedure increases consumption of ligand, it removes the requirement that ligand is not damaged by regeneration and allows the ligand to be changed between analysis cycles on the same sensor chip.
- Capture may be used to attach a specific ligand from a complex sample, provided the capturing interaction is sufficiently selective. Covalent immobilization of ligand from such samples would require prior purification of the ligand.

Conditions for ligand capture are dictated by the requirements of the capturing interaction, and capturing is normally performed in the same buffer conditions as the interaction being studied. It is however important that the capturing interaction is reasonably stable under the chosen conditions, so that ligand does not dissociate significantly during sample analysis. Demands on capturing stability vary with different applications, and are highest for kinetic and affinity analyses, since loss of ligand will cause a fall in response as well as reducing the analyte binding capacity of the surface. Simpler applications such as detection and screening may tolerate more loss of ligand during sample injection.

3.2.3 Response levels

One of the issues in preparing the sensor surface for a given application is the question of how much ligand that should be attached. All steps in the attachment process are monitored in the instrument, and the amount of attached ligand is in principle given directly by the response above baseline (Figure 3-3).

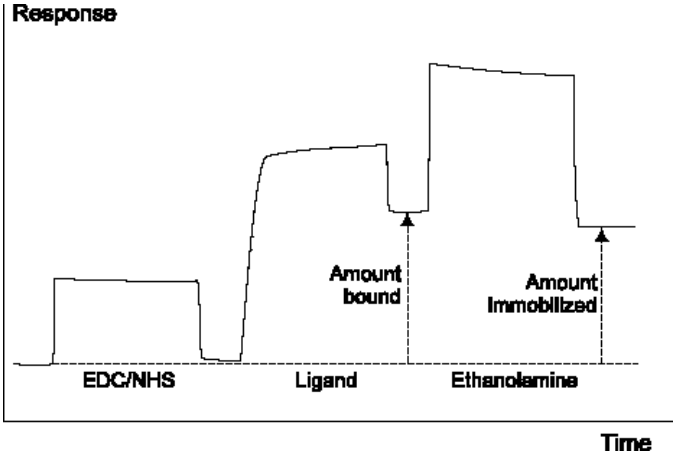


Figure 3-3. Sensorgram from a typical amine coupling, illustrating the distinction between the amount of ligand bound and the amount immobilized.

Note: *Even activation of the surface with EDC/NHS can be detected as a small increase in response. When the response from immobilized ligand is low, it may be more appropriate to estimate the ligand level from the response after activation rather than the baseline at the start of the immobilization procedure.*

A more relevant parameter in designing and preparing a surface is however the analyte binding capacity (called $_{Rmax}$, for maximum response), since this will determine the expected response range from samples. The amount of ligand on the surface as measured from the increase in response gives an indication of the *theoretical* analyte binding capacity of the surface, provided that the relative sizes of ligand and analyte are known. Assuming a 1:1 binding stoichiometry:

$$R_{max}\,(RU) = \frac{\text{analyte MW}}{\text{ligand MW}} \times \text{immobilized ligand level (RU)}$$

The practical analyte binding capacity is obtained experimentally by injecting analyte at a high concentration to saturate the surface (or by extrapolating binding levels from measurements at a series of concentrations). Comparison of the theoretical and practical R_{max} values provides a measure of the activity of surface-attached ligand. Protein preparations are seldom 100% active, and for generic attachment methods such as amine coupling, activities in the region of 70% and above may be expected.

The shape of the sensorgram from the attachment procedure can provide guidance in choosing appropriate conditions. Bear in mind that the amount of ligand immobilized will generally be less than the amount bound during preconcentration. Most Biacore systems support a mode of ligand immobilization using amine coupling that exploits the information from a brief

preconcentration injection to adapt the immobilization process to reach a specified level.

- The sensorgram slope and shape during protein injection indicates the efficiency of electrostatic preconcentration. Try to use a combination of ligand concentration and injection time so that the amount bound reaches a plateau. This will contribute to the robustness of the immobilization procedure.
- The relative response levels before and after deactivation of the surface indicate how well the electrostatically bound protein has become covalently attached. Deactivation washes out non-covalently bound material: a reduction in response may be observed after deactivation but this does not affect the assay as long as sufficient ligand is attached to the surface.

If the immobilization works in principle satisfactorily but the amount of ligand attached is not suitable for the assay, adjust one or more of the parameters ligand concentration, immobilization pH, ligand injection time and surface activation time.

3.3 General buffer considerations

3.3.1 Buffer substances

In general, Biacore systems are compatible with most buffer substances used in biological studies. Exceptions may be dictated by specific properties of the interacting molecules or by the situation where the buffer is used (for example, the primary amine groups in Tris preclude the use of this buffer for attaching ligand by amine coupling).

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pK_a 7.55) is a satisfactory buffer substance for many protein-protein interactions, and ready-to-use buffers based on 10 mM HEPES are available for use with Biacore systems.

Phosphate-based buffers are often more suitable for work with low molecular weight analytes since organic buffers such as HEPES can bind to the ligand and interfere with accurate detection of low molecular weight compounds.

3.3.2 Ionic strength

Physiological ionic strength buffers (containing 0.15 M monovalent cations) are recommended unless other requirements are imposed by the interaction being studied. Reducing the ionic strength will increase the tendency for non-specific electrostatic binding of molecules from the sample to the sensor surface. Higher ionic strength may be tested if non-specific binding is a problem.

3.3.3 Additives

Detergent

Inclusion of non-ionic detergent (0.05% Surfactant P20, Tween™ or equivalent) in all buffers is recommended to minimize deposition of protein and other biomolecules in the flow system. The detergent should be included at a concentration above the critical micelle concentration (CMC). At lower concentrations, the detergent itself can accumulate in the flow system and lead to unwanted response effects as it is released (see FIgure 3-4).

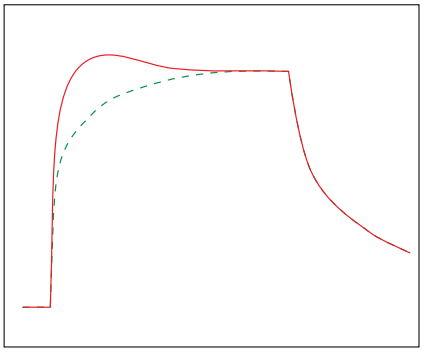


Figure 3-4. Schematic illustration of typical "detergent effects" caused by using detergent at concentrations below CMC. The broken line shows an undisturbed sensorgram for comparison.

Some applications, particularly those involving lipid vesicles and/or hydrophobic proteins, require detergent-free buffer. In such cases it is essential to clean the flow system regularly and carefully using the appropriate maintenance tools provided with the system, in order to ensure continued high performance. Poor instrument cleaning can result in severely disturbed sensorgrams that are difficult to interpret. A severe example is shown in Figure 3-5.

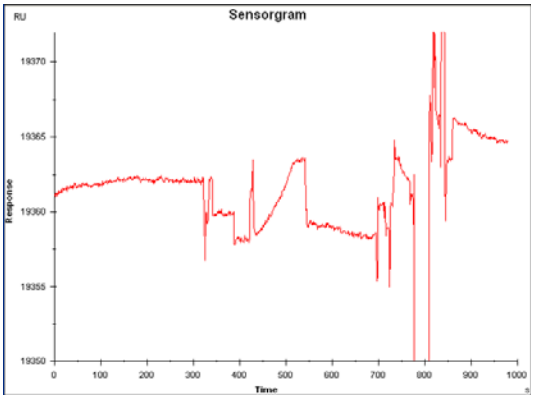


Figure 3-5. Severe example of the consequences of poor instrument cleaning.

EDTA

HBS-EP and HBS-EP+ general purpose buffers from GE Healthcare include 3 mM EDTA to remove traces of free divalent metal ions that may be present in the buffer. This is a general precautionary measure and is not specifically required for work with Biacore systems. Buffers with no added EDTA are also available.

NSB Reducer

Addition of NSB reducer (a preparation of soluble carboxymethyl dextran) to samples can in some cases help to reduce non-specific binding of sample components to the dextran matrix on the sensor surface. The recommended concentration of NSB Reducer is 1 mg/ml. NSB Reducer should only be used in situations where non-specific binding is a problem (typically with complex samples such as serum or whole cell extracts).

Organic solvents

Many low molecular weight organic compounds, relevant particularly in pharmaceutical development work, are sparingly soluble in aqueous buffers and require addition of organic solvents. Dimethyl sulfoxide (DMSO) is commonly used. Biacore systems can be used with buffers containing up to 10% DMSO. However, organic solvents contribute significantly to the bulk refractive index of samples and buffers: 1% DMSO contributes approximately 1200 RU to the response level. Expected responses from low molecular weight analytes, which often need organic solvents to maintain solubility, may be as low as 10-20 RU or less. It is therefore crucial that the measured responses are accurately compensated for any variations in organic solvent concentration. Procedures for adjusting measured sample responses for solvent effects, called *solvent correction*, are described in principle in Appendix B and in more detail in the documentation for Biacore systems where the correction is supported.

Other additives

Avoid additives that can compete with the ligand for immobilization chemistry, for example sodium azide with amine coupling chemistry. Even at low concentrations, sodium azide can effectively prevent amine coupling of proteins.

3.3.4 Buffer preparation

All buffers regardless of composition should be filtered before use and degassed if the instrument does not have a built-in degasser. Disturbances caused by particles and microscopic air bubbles introduced over the sensor surface appear typically as spikes in the sensorgram. Air bubbles or particles that remain on the surface can result in more long-lived displacements in the response (Figure 3-6).

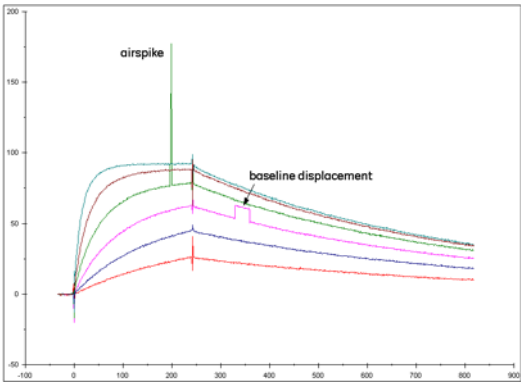


Figure 3-6. Airspikes and transient baseline displacements are often attributable to inadequately degassed running buffer.

Filtering

Buffers should be filtered through a 0.22 µm filter to remove particles.

Serum and plasma samples should be centrifuged or filtered to remove aggregated material, particularly if the samples have been frozen.

Degassing

Degas running buffer before use unless you are working with a Biacore system fitted with an in-line degasser. Alternatively use degassed ready-to-use buffers from GE Healthcare.

Always degas buffer solutions before adding detergent to avoid frothing.

3.4 Matching sample and running buffer

3.4.1 Matching refractive index

Differences in refractive index between sample and running buffer give rise to so-called bulk shifts in sensorgrams at the beginning and end of sample injection. The bulk shift may be negative or positive depending on the difference in refractive index (Figure 3-7).

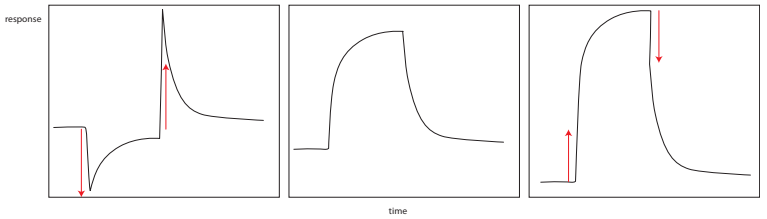


Figure 3-7. Schematic illustration of bulk shifts: negative (left), zero (center) and positive (right).

Some applications, such as concentration measurement and in some cases screening, rely on single-point measurements (report points) that can be placed after the end of the sample injection. These measurements are not affected by bulk shifts so that accurate matching sample and running buffer is not necessary (Figure 3-8).

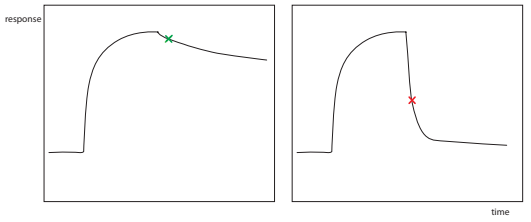


Figure 3-8. If dissociation is slow enough, binding levels can be assessed from a report point placed shortly after the end of the sample injection (left). However, if dissociation is rapid (right) a report point at this position will not give a reliable measure of the binding level.

In contrast, applications that rely on response measurement during sample injection need to deal with the question of bulk response. Careful matching of sample and running buffer with respect to refractive index goes a long way to eliminating bulk shifts, but exact matching is seldom practicable. Subtracting the response from a reference surface usually handles bulk shifts satisfactorily provided that the shifts are reasonably small in relation to the measured analyte responses. Special measures, called solvent correction, are however required for applications where bulk shifts can be large and expected responses are small (typically assays involving low molecular weight organic analytes, such as

screening for potential drug development candidates). These special considerations are dealt with in Appendix B.

It is not always possible to match the refractive index of sample and running buffer, for example in analysis of unpurified samples such as serum. In such cases it is important that measurements are made after the end of the sample injection to avoid bulk shift effects.

3.4.2 Matching buffer composition

Matching of sample and running buffer composition is particularly important for kinetic measurements, regardless of the question of bulk response shifts, since the sensorgram contains information about dissociation kinetics both during and after sample injection. For correct analysis of the kinetics, it is important that the dissociation process during and after sample injection occur in the same environment.

3.4.3 Matching buffer environment in practice

Several approaches can be used to match sample and running buffer, depending on the form in which the original sample is available.

- Samples that are available in sufficiently concentrated stock solution may be diluted in running buffer so that the concentration of other components in the stock is negligible. The minimum dilution factor needed to achieve this depends on the composition of the stock solution and the stringency of the buffer matching requirements for the assay at hand.
- Buffer exchange techniques such as desalting columns or dialysis are often the most effective way to prepare samples in running buffer. Buffer exchange can be essentially complete with minimum dilution of the sample, and commercially available solutions such as micro-spin columns allow rapid preparation of small sample volumes.
- Solid samples may be dissolved directly in running buffer: filter or centrifuge the solution if there is a risk that undissolved material remains. Be aware however that many commercially available proteins supplied in solid form include considerable amounts of salt or other stabilizing medium. Dissolving a dry preparation in running buffer does not guarantee that the composition or refractive index of sample and buffer will be matched.

3.5 Dilution series and replicates

Recommendations in this section relate to factors that should be standard in laboratory work. They are however included here as a reminder, since they can in some instances be critical to the success of the application.

Dilution series play an important role in a number of contexts in work with Biacore systems: for example, kinetics and affinity determinations require analysis over a range of analyte concentrations, and calibrated concentration assays use dilution series for establishing a standard curve and also for investigating certain performance characteristics such as parallelism. In many situations, performing replicate determinations to establish reproducibility is a given component in assay design. The way in which dilution series and replicates are prepared and analyzed can have a significant impact on the value of the results.

3.5.1 Dilution series

There are various possible sources of error in creating a dilution series, and the best approach depends on the available equipment and the volumes involved as well as the accuracy demands of the application.

Reliable pipettes are essential for creating accurate dilution series by volume. Where possible (particularly for small volumes), use repeated aliquots of the same volume for dilution series, to avoid errors that may be introduced by repeatedly changing the volume on variable volume pipettes.

Two different basic methods may be used:

- Serial dilution where each dilution is used as the sample solution for the next step.
- Parallel dilution where sample is always taken from the same stock solution and mixed with increasing volumes of buffer.

Using the same fixed volume pipettes for both sample and buffer provides the best accuracy for dilution factor (although not necessarily for absolute volumes). If different pipettes are used with different errors of delivery, the dilution error will propagate through the series more significantly with serial dilution than with parallel. However, serial dilution is a simpler procedure, requiring fewer operations with a fixed-volume pipette, so that parallel dilution is more susceptible to errors of reproducibility.

For the best accuracy in preparing dilution series, volumes of buffer and sample used may be determined by weighing and the dilution factor calculated individually for each sample. This avoids issues of both calibration accuracy and reproducibility for pipetted volumes.

3.5.2 Replicates

Measuring replicate samples to establish the reproduciibility of an assay should be a standard procedure in all laboratory work, but it is not always clear how the replicates should be prepared and measured in order to provide the most useful information. Replication can cover the whole assay procedure from raw sample material to evaluation of the final results, or it can focus on one or more specific

components of the procedure. Different assay situations will require different approaches in this respect.

Replicate samples designed to test the reproducibility of a Biacore-based assay should be prepared and measured according to the following general guidelines:

- Take replicates from a well-defined step in the sample preparation procedure, with a clear awareness of what parameters are being tested for reproducibility. For example, taking replicate samples from the same tube in a dilution series will test the reproducibilty of the assay procedure itself, whereas preparing replicate dilution series from a common stock solution will include pipetting and dilution procedures in the repliacte test.
- Place replicate samples in separate vials or microplate wells for the assay wherever possible, so that only one sample is taken from each position. Injecting multiple samples from the same autosampler position is not recommended since evaporation from vials or wells that have been penetrated can affect the concentration.
- Analyze replicates at well-spaced intervals in the assay, to provide a check on any drift in the assay performance with time. In many assays, this factor can be conveniently tested by using repeated measurements on control samples: in some cases however (such as kinetics and affinity analysis) it can be valuable to apply the principle to replicate concentrations of the sample.

3.6 Preparing vials and microplates

Samples and reagents are loaded into sample racks or microplates (according to the requirements of the particular Biacore instrument). All samples for one assay run are normally loaded at the start of the run: if samples have a tendency to precipitate with time, bear in mind that the samples that are analyzed last may have been standing for several hours in the autosampler.

Beware of using samples that might precipitate or aggregate with time (for example, low molecular weight organic compounds close to the limit of solubility).

Cover samples as soon as they are prepared, and keep them covered for the duration of the assay to prevent evaporation. Always use vial caps and microplate foil for Biacore systems from GE Healthcare: the autosampler needle may not penetrate caps or foil from other sources satisfactorily, resulting in failed assays or even damage to needle. When sealing microplates with foil, make sure the foil is correctly placed so that the wells are covered by areas free from adhesive. If the foil is wrongly applied, adhesive may accumulate on the needle and cause malfunction.

4 Screening and detecting binding partners

Screening applications using Biacore systems fall into two main categories that differ significantly in challenges and experimental design:

- Small molecule (LMW) screening, where response levels are low and organic solvents are often needed to maintain analyte solubility. These interactions are usually rapid and regeneration is not needed.
- Biopharmaceutical (frequently antibody) screening where response levels are higher but the sample matrices (cell culture supernatant or cell extracts) are often complex. These interactions are usually slower: regeneration is simplified if a capture approach is used.

Table 4-1 summarizes the main experimental conditions for LMW and antibody screening.

Table 4-1. Main experimental conditions for LMW and antibody screening.

	LMW screening	**Antibody screening**
Buffer	Phosphate	HEPES
Additives: - DMSO - detergent	 Yes (1 to 10%)[1] Yes	 No Yes
Flow rate	Typically 10 µl/min	Typically 10 µl/min
Contact time	15 to 30 s	1 to 2 min
Report point	Before end of sample injection	After end of sample injection
Regeneration	Usually not needed	Usually needed

[1] Recommended 2% unless higher concentration is required to maintain substance solubility.

A third related application area is immunogenicity testing, which involves detecting anti-drug antibodies (ADAs) in plasma or serum samples. Here the simultaneous presence of ADAs and drug in the samples creates special requirements.

4.1 Small molecule screening

4.1.1 Goals

The primary goal of small molecule screening in pharmaceutical development is to identify candidate molecular structures for further development, on the basis of their binding to selected target molecules. The first stages of the process is often screening of candidate libraries or fragment libraries to identify promising binders for further development and refinement. Information-rich screening with Biacore systems can provide a wealth of valuable data in addition to yes/no binding results, including comparative binding to multiple targets to identify promiscuous (and therefore less interesting) binding candidates and assessment of kinetics and affinity properties that become increasingly important as the candidate molecules are refined.

4.1.2 Sensor surface preparation

The response obtained from LMW analytes is inherently low because of the molecular size: in addition, binding affinities are often weak (particularly for fragment screening) which further reduces the expected response levels. For this reason, sensor surfaces for LMW screening are prepared with high levels of ligand (typically 8,000 to 10,000 RU for average-sized proteins).

Accurately determining the activity of the immobilized ligand requires high analyte concentrations to saturate the surface with weak binders (typically 0.1 to 2 mM for fragments and 0.05 to 0.2 mM for LMW compounds). Testing binding activity with a positive control is desirable if a suitable control analyte is available.

4.1.3 Sample preparation

In the first phases of small molecule screening, samples are often prepared at a fixed concentration in the same buffer. There is usually no opportunity to optimize concentration or buffer conditions.

Many small organic compounds are sparingly soluble in aqueous buffers and require inclusion of organic solvents (commonly 1% to 3% DMSO) to maintain solubility. DMSO has a strong refractive index contribution (about 1200 RU for 1% DMSO), and both careful matching of sample and running buffer and correction for small differences in DMSO concentration are required (see below).

4.1.4 Buffers

Phosphate buffers are generally recommended for work with small molecules. Using organic buffers such as HEPES can bind to the ligand and interfere with accurate detection of small organic compounds. Physiological ionic strength (150 mM monovalent cations) should be used to reduce non-specific binding of

compounds to the sensor surface, and inclusion of detergent (0.05% Surfactant P20) generally improves data quality by reducing drift and signal disturbances.

The composition of running buffer and sample buffer must be matched as closely as possible, particularly with respect to organic solvent concentration. Variations in DMSO content between samples resulting from both evaporation and absorption of water are however unavoidable. Procedures that correct for differences in bulk refractive index between samples, called *solvent correction*, are described in Appendix B and are implemented in Biacore systems designed for work with small molecules.

4.1.5 Analysis conditions

Flow rate

Use low flow rates (10 µl/min) for LMW screening applications to conserve sample. Issues such as mass transfer limitations (Section A.1) and resolution of fast binding events are not relevant.

Contact time

Most interactions involving small molecules are rapid, and contact times can be kept short (30 to 60 seconds) to increase screening throughput.

Regeneration

Regeneration is generally not necessary in LMW screening applications, since dissociation is rapid and is usually complete within 1 minute or thereabouts.

Report points

Because of the rapid dissociation of many LMW compounds from the target molecule, report points for screening should be placed before the end of the sample injection. LMW compounds sometimes show rapid binding to specific sites followed by slower and more indiscriminate binding (Figure 4-1). Placing report points early in the sample injection can reduce the impact of this behavior and give a more accurate indication of specific binding ability.

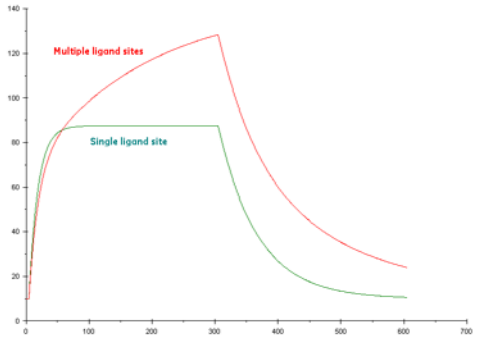


Figure 4-1. Simulated sensorgrams illustrating 1:1 binding (dark green) and binding to one specific site followed by slower and more indiscriminate binding (red).

Other considerations

Some LMW compounds are “sticky” and can be difficult to wash off the sensor surface and out of the flow system, causing carry-over of material to the next analysis cycle. This can be detected by routinely including a “carry-over injection” of buffer after the sample injection: the response from a “sticky” compound will be carried over into this buffer injection. Include a flow system wash with 50% DMSO after every cycle to minimize problems with sticky compounds. If a screening experiment involves compounds that are known to be sticky, place these at the end of the experiment to avoid interference with other compounds or omit them from the experiment altogether.

4.1.6 Evaluating results

At the simplest level, LMW screens are evaluated by comparing the binding responses corrected for analyte molecular weight, and setting a cut-off level above which binding is regarded as significant. The cut-off may be determined by comparison with the response obtained from known non-binders (negative controls) or set to some level chosen in relation to the distribution of response values.

A valuable way to compare kinetic properties is to plot the association rate constant against the dissociation rate constant on logarithmic axis. Diagonal lines in this plot represent constant affinity, so that compounds that lie on the same diagonal but are separated from each other have the same affinity but different kinetics (Figure 4-2).

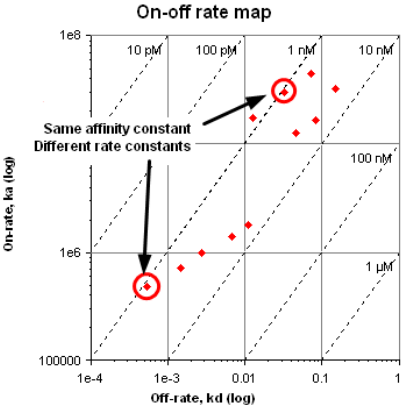


Figure 4-2. Plotting association rate constant k_a against dissociation rate constant k_d (here on logarithmic scales) creates a plot where the affinity is represented by diagonal lines. Compounds on the same diagonal have the same affinity but differ in kinetics.

Fragment screening benefits from additional steps in experimental design and evaluation, to identify and characterize interesting fragments. In summary, the recommended approaches are:

- Clean screen, aimed at rapidly identifying and eliminating undesirable "sticky" compounds that show persistent binding to the surface and can disturb subsequent analysis cycles.
- Binding level screen, aimed at providing a rapid overview of the compound library, identifying compounds with binding levels above a defined cut-off.
- Affinity screen, aimed at verification of binding and affinity ranking of fragments.

These approaches are described in more detail in the handbooks for Biacore systems that support fragment screening.

4.2 Antibody screening

4.2.1 Goals

The goal of antibody screening is to identify cell clones that produce antibodies appropriate for the purpose (usually biopharmaceuticals or biochemical tools). Screening needs to be rapid so that clones can be selected before they grow to unsuitable cell densities. Obtaining kinetic information early in the screening process is often a significant advantage. Information that is generally sought includes:

- Which clones produce antibodies with suitable specificity
- Which antibodies have kinetic and/or affinity properties suitable for the purpose
- Which of these clones produce antibodies in sufficient amounts

4.2.2 Sensor surface preparation

Unlike LMW screening, expected response levels in antibody screening can be quite high, so there is no need to use high levels of immobilized or captured ligand. Typically, sensor surfaces are prepared with a generic anti-antibody such as anti-IgG for which assay conditions (including regeneration) are known, and binding characteristics are determined with an injection of antigen after antibody capture. It is also possible to perform an antibody screen directly with antigen attached to the surface as ligand, but this approach often involves additional work to establish regeneration conditions.

4.2.3 Sample preparation

Samples for antibody screening are typically taken from cell culture supernatant and analyzed directly without purification. Preliminary experiments with non-producing clones to establish that there is no significant binding of non-antibody components can simplify interpretation of the results.

4.2.4 Buffers

HBS-EP+ (Hepes-buffered saline with 0.3 mM EDTA and 0.05% Surfactant P20, available from GE Healthcare) or similar is recommended as running buffer. Samples may be diluted if required in the same buffer. Precise matching of sample and running buffer is not practicable for screening work.

4.2.5 Analysis conditions

Flow rate

Antibody screening by capture on a generic capturing molecule can be run at low flow rates (typically 10 µl/min) if sample consumption is an issue. Characterization of the antibody-antigen interaction, whether performed directly on immobilized antigen or by a secondary injection over captured antibody, should be run at moderate flow rates (typically 30 µl/min) to reduce mass transfer limitations (see Section A.1).

Contact time

Contact times should be sufficient to give confidently measurable response levels without compromising screening throughput. Contact times of 1 to 2 minutes are usually sufficient.

Regeneration

Regeneration conditions for antibody screening kits from GE Healthcare are specified in the respective Instructions for Use. For assay development using custom antibodies, regeneration at low pH (glycine-HCl, pH 1.5 to 3) is usually effective.

Report points

For screening, place a report point shortly after the end of the sample injection. This will avoid any bulk refractive index differences between sample and running buffer.

Antibody characterization based on complex stability uses report points placed early and late in the dissociation phase of the antibody-antigen interaction.

4.2.6 Evaluating results

The response reached in the first step of the screening gives an indication of the relative concentration of antibodies in the sample (according to the specificity of the capturing molecule). Responses achieved from injection of antigen reflect both the antigenic specificity and the binding characteristics of the captured antibody. Note however that evaluation based on report points alone does not give unambiguous information: a given response may be obtained from a combination of high concentration and slow binding or low concentration and fast binding. It may be possible to resolve these alternatives by visual inspection of the sensorgrams, but if the binding and dissociation events are rapid both situations may give closely similar sensorgrams (see Figure 4-3).

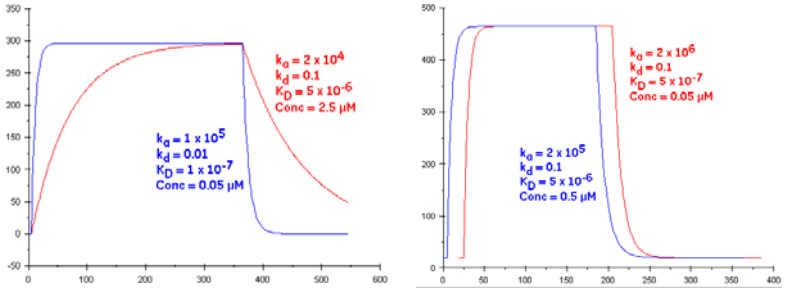


Figure 4-3. Sensorgram shape can in some cases distinguish weak and strong binders that reach the same response level as a result of different concentrations (left). In other cases, depending on the relative values of the rate constants and concentrations, the sensorgrams may be identical. These sensorgrams are simulated: those in the right-hand panel are displaced slightly from each other on the time axis.

Frequently, the parameter of most interest in choosing between antibody clones is the stability of the antibody-antigen complex, which is largely governed by the dissociation rate. Comparison of the response early and late in the dissociation phase can give a rapid indication of the relative dissociation rates.

4.3 *Immunogenicity testing*

4.3.1 Goals and challenges

Immunogenicity testing answers the question of whether administered drugs provoke an immune reaction in the recipient that can compromise the efficacy of the drug. At a simplistic level, this application is analogous to screening for antibodies in serum, and recommendations for screening apply also to immunogenicity testing, using immobilized drug as ligand. However, the simultaneous presence of the drug and circulating anti-drug antibodies (ADAs) in serum presents a special challenge: ADAs in complex with drug will not be detected in an assay based on binding to the drug.

ADAs are released from drug complexes under acidic conditions (pH 1 to 2), and can be detected if the sample is neutralized and the assay is performed immediately, before the complexes have had time to re-form fully. This approach is implemented in some Biacore systems, using a specially designed IFC that mixes acidified samples with neutralizing solution immediately before injection over the sensor surface. Details of the approach are described in the manuals for systems that support immunogenicity testing (e.g. *Biacore T200 Immunogenicity Handbook*).

5 Kinetics and affinity measurements

5.1 Approaches

Determination of both kinetics and affinity rely on measurements over a range of analyte concentrations. Kinetic (rate) constants are obtained by analyzing the sensorgram shape in relation to a mathematical model of the interaction mechanism: affinity constants are obtained from the steady state binding levels. Affinity constants can also be calculated from the ratio of the rate constants. Details of the data analysis principles are given in Appendix A.

5.1.1 Single- and multi-cycle kinetics

There are two approaches to determination of kinetics and affinity, both based on determination of binding data at several (typically 5) analyte concentrations (see Figure 2-2):

- *multi-cycle kinetics*, where each analyte concentration is injected in a separate analysis cycle, and the sensor surface is regenerated between cycles. Since data from the different concentrations is evaluated together, it is important that regeneration is carefully optimized so that the surface properties are constant between cycles.
- *single-cycle kinetics*, where analyte concentrations are injected sequentially in a single cycle, with no regeneration between injections.

Both approaches have been shown to give equivalent results in test systems, and both can be used for steady-state affinity measurements if the sample injection time is long enough for steady state to be reached.

The single-cycle approach requires less time for a complete analysis, and also benefits from the lack of requirement for optimized regeneration. This can significantly reduce the demands on assay development, and permits kinetic determinations for interactions where regeneration conditions are not available. However, the cycle time is longer in single-cycle kinetics, so the approach is more sensitive to drift such as dissociation of ligand in a capture assay.

5.1.2 2-over-2 kinetics

Normally, all samples are analyzed over a single sensor surface, providing identical ligand conditions for each analyte concentration. In Biacore A100 and Biacore 4000 systems, however, parallel analysis over multiple ligand spots in each flow cell permits a technique called *2-over-2 kinetics*. The name refers to analysis of two analyte concentrations over two ligand densities, and can be performed in a single cycle in Biacore systems which can support multiple

ligand densities in each flow cell. Combining the data from two ligand densities compensates for the lack of analyte concentrations, and the approach can provide reliable kinetic data from experiments that maintain a moderately high sample throughput.

5.1.3 Comparative estimates of kinetics and affinity

Comparison of relative interaction kinetics can be sufficient for some applications. At the simplest level, this can be achieved by plotting the ratio of response values early and late in the sample injection (for association rates) and in the dissociation phase (for dissociation rates), using a single analyte concentration. This simplified approach is sometimes used in information-rich screening work, where full determination of kinetics and/or affinity is not practicable for throughput considerations.

5.2 Sensor surface preparation

Amount of immobilized ligand

In evaluation of kinetic and affinity constants, the concentration of ligand is expressed in resonance units (RU) so the amount of immobilized ligand does not have to be known exactly (see Appendix A). The immobilization level is however important for the quality of the results.

For kinetic analysis, the amount of immobilized ligand should be kept low, so that the maximum response from analyte binding is typically in the region of 30 to 50 RU or lower. There are two main reasons for this:

- Low ligand levels help to reduce limiting effects of mass transfer. Mass transfer refers to the diffusion-controlled supply of analyte molecules to the surface from bulk solution: if transfer is slow in relation to the association rate, the observed binding will be a measure of the diffusion process and not of the interaction rates. Mass transfer is discussed in more detail in Appendix A.
- Low ligand levels also help to minimize artifacts that can arise from crowding of molecules on the surface. Crowding effects are difficult to quantitate but can give rise to unnecessarily complex binding behavior.

For measurement of steady-state affinity, mass transfer considerations are not relevant and the amount of immobilized ligand can be higher than for kinetic analysis.

Reference surface

Kinetic and affinity measurements should always use reference-subtracted data. The reference surface may be untreated, activated and deactivated, or prepared with an inactive protein to mimic the physical properties of the active

surface. These alternatives are discussed in more detail in the Biacore Sensor Surface Handbook.

5.3 Buffers

The same buffer must be used for sample and running buffer, so that association (during sample injection) and dissociation (after the end of the injection) occur in the same environment. Moreover, evaluation of kinetics is more robust if the sample and running buffer are matched in refractive index, so that bulk shifts at the beginning and end of the sample injection are kept as small as possible.

Buffer matching is less important for affinity measurements since evaluation is based only on response values measured during the sample injection. Bulk response contributions will however introduce an offset from the x-axis in the plot of response against analyte concentration (Figure 5-1). It is important that bulk effects do not vary between samples so that the offset is constant.

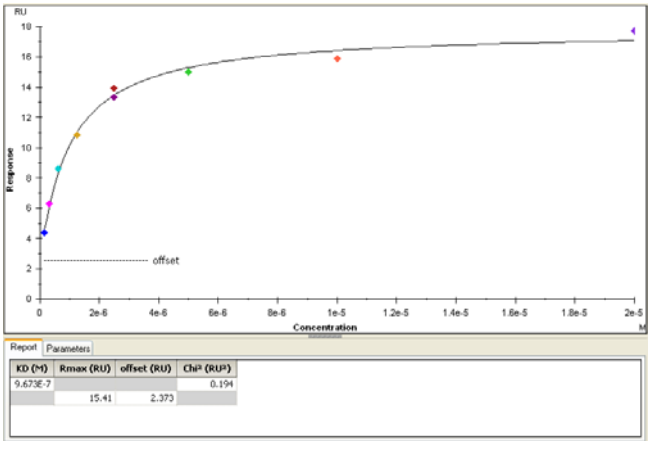


Figure 5-1. Plot of response against analyte concentration evaluated with offset to allow for bulk effects.

5.4 Sample preparation

Preparation of samples can be a critical factor in the quality of kinetic measurements. Ideally, both ligand and analyte should be pure and homogeneous, to simplify the interpretation of the results as far as possible. Using a capturing approach for attaching ligand provides on-chip purification: however, it is essential for careful work that the capturing interaction is sufficiently stable so that ligand does not dissociate during the sample analysis. It is important that non-specific binding of sample components to the sensor surface is kept to a minimum. It is also important that the analyte is

homogeneous and non-aggregated since differently sized binding species will give different responses. Use non-denaturing analysis methods to check analyte homogeneity.

Samples may be diluted into running buffer from stock solution if the dilution factor is sufficiently high so that residual stock solution components can be ignored. Alternatively, samples may be prepared using buffer exchange techniques.

5.5 Sample concentrations

Evaluation of association rate constants and affinity constants from the experimental data is directly dependent on the values provided for analyte concentrations (see below for details). For this reason, it is important for obtaining correct results that the analyte concentration is determined as accurately as possible. Ideally, this should be the concentration of analyte that is capable of interacting with ligand, which may not be the same as the total concentration (if for example the analyte is only partially active). Calibration-free concentration analysis (see Section 2.3.2) provides an approach to concentration measurement that is directly related to the amount of analyte that can bind to the ligand, and is recommended in critical measurements. If there are doubts about the validity of the analyte concentration values, the reported values for the association rate constant and affinity constant should be treated with due caution. The dissociation rate constant is not dependent on the analyte concentration.

Analyte concentration range

In principle, kinetic constants can be obtained from analysis at a single analyte concentration, but analysis over several concentrations (5 to 8 for multi-cycle kinetics, up to 5 for single-cycle kinetics) gives a better foundation for discovering deviations from the simplest interaction models and for revealing concentration-dependent artefacts (for example analyte aggregation at increasing concentrations). Ideally, concentrations should cover a wide range centered on the value for the dissociation equilibrium constant K_D (for example, for an interaction with K_D = 10 nM, concentrations from 1 to 100 nM are appropriate). In practice, the K_D value is often not known, and the range of concentrations that can be used may be limited by availability or solubility of the analyte. A concentration range that gives a good spread of sensorgrams is usually adequate (Figure 5-2).

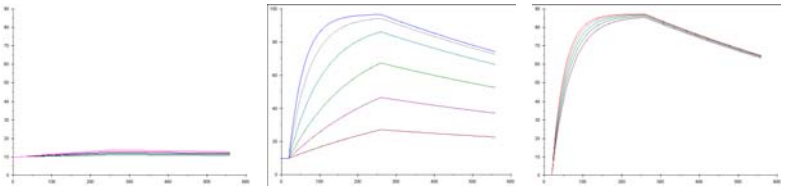


Figure 5-2. Analyte concentrations for kinetic analysis should cover a wide range (center panel). If the concentrations are too low (left panel) or too high (right panel) the sensorgrams may be tightly grouped and evaluation will be less robust. The vertical scale for these simulated sensorgrams is the same in all three panels.

For affinity determinations, each analyte concentration gives rise to a single point on the experimental data plot, in contrast to kinetic analysis where each analyte concentration provides a full time-course of the interaction. It is therefore important to use a sufficient number of analyte concentrations in the assay (the recommended minimum is 5). In addition, the concentration range should extend to more than twice the affinity constant (K_D) value. If these conditions are not fulfilled, there will be insufficient data in the plot of R_{eq} against C for confident analysis. Conversely, a reported K_D value higher than half the highest analyte concentration should be treated with care (Figure 5-3).

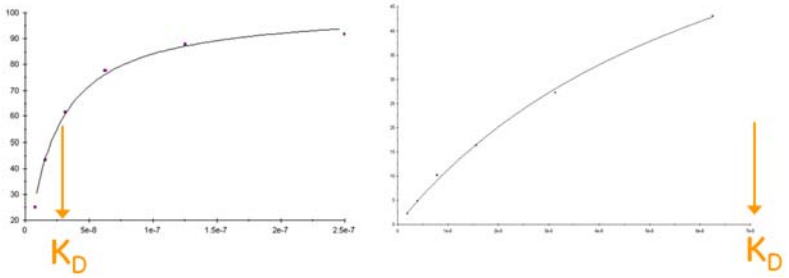


Figure 5-3. The analyte concentration range for steady state affinity determination should cover more than twice the K_D value (left). Do not trust reported affinity constants that are higher than half the highest analyte concentration (right).

Analyte concentration values

The association rate constant for a simple binary interaction has units of $M^{-1}s^{-1}$ (see Appendix A), and molar analyte concentrations must be supplied to the software for kinetic evaluation. Similarly, evaluation of steady state affinity requires analyte concentrations as input. It is important that analyte concentrations are correct if correct values for rate constants are to be obtained. Incorrect values for analyte concentration will lead directly to corresponding errors in the association rate constant k_a.

Kinetic measurements rely on analysis over a range of analyte concentrations, usually prepared by serial dilution from a stock solution. Properly calibrated

equipment and well-designed sample preparation protocols are essential if the concentrations are to be correct (see Section 3.5.

In addition to accurate sample preparation, correct values for the stock solution concentration are important. The ideal value is the concentration of analyte that is capable of binding to the surface-attached ligand: this may not necessarily be the same as the total analyte concentration. If half of the analyte molecules are in a conformation that cannot bind, the total concentration will be twice the value that should be used for evaluation of binding kinetics. If the sample is amenable to concentration determination by CFCA, this is the value that should ideally be used: it represents the concentration capable of interaction and is independent of calibration considerations. Figure 5-4 illustrates an example of the importance of using the correct analyte concentration.

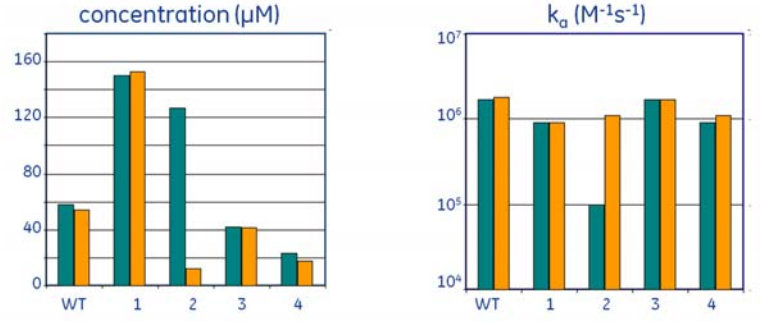


Figure 5-4. Importance of using the correct analyte concentrations. The experiment compared the kinetics of wild type and four mutants of a papain inhibitor binding to papain. Using concentrations obtained from A_{280} measurements (green bars), mutant number 2 had an association rate constant about one order of magnitude lower than the wild type and the other mutants. However, using concentrations obtained by CFCA (orange bars), association rate constants obtained were similar for all variants of the inhibitor.

6 Epitope mapping

Epitope mapping refers to determination of the relative topography of binding sites (usually for antibodies) on a target molecule or antigen. Antibodies that bind to epitopes that are distinct from each other can bind simultaneously to the antigen, while antibodies directed against the same or interfering epitopes show mutually exclusive binding.

The commonest approach to epitope mapping is to test simultaneous binding ability in a pair-wise matrix. In an alternative approach, peptides representing structural epitopes on the antigen are tested for inhibition of antibody binding. Both methods are amenable to use with Biacore systems.

6.1 Pair-wise binding

6.1.1 Principle

The basic principle of pair-wise mapping studies in Biacore systems is straightforward: one antibody is attached to the surface, antigen is bound to the antibody, and a second antibody is tested for simultaneous binding ability. In practice, the assay set-up involves additional steps (see Figure 6-1):

- The first antibody to the sensor surface is conveniently attached to the sensor surface by capturing on a generic antibody such as rabbit anti-mouse immunoglobulins for mouse antibodies. This allows multiple antibody pairs to be tested using the same sensor surface. Permanent attachment is less suitable since the first antibody cannot then be changed without preparing a new sensor surface.
- It is important to ensure that no antibody-capturing sites on the surface are available to bind the second antibody when it is injected. Remaining capture sites are therefore blocked with an injection of blocking antibody (an antibody that does not bind to the antigen being tested).
- Antigen is then injected and binds to the first antibody.
- The antigen is followed by an injection of second antibody to test for simultaneous binding of the two antibodies.
- The surface is regenerated to remove all components except the permanently attached capturing antibody, in preparation for testing the next antibody pair.

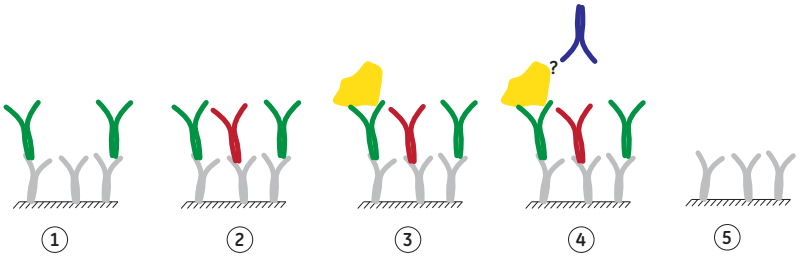


Figure 6-1. Main steps in a pair-wise epitope mapping experiment.
1. Capture first antibody.
2. Block remaining capture sites.
3. Inject antigen.
4. Challenge with second antibody.
5. Regenerate in preparation for the next antibody pair.

A panel of *n* antibodies contains *n(n-1)/2* unique pairs of non-identical antibodies (for example 5 antibodies give 10 pairs). It is recommended to test all combinations in the pair-wise matrix:

- Pairs where the first and second antibody are identical provide a check on the assay performance. If simultaneous binding is observed this may indicate that the antigen contains multiple copies of the epitope, which may invalidate the mapping with respect to that epitope.
- Pairs where the roles of first and second antibody are reversed provide a control for unexpected effects such as conformational changes induced by antibody binding. Ideally, the results should be independent of the order in which antibodies are tested, but this is not always the case in practice.

6.1.2 Evaluation

The label-free detection in Biacore systems offers advantages over label-based methods for epitope mapping, in that all steps in the binding sequence are monitored with the same technique. It is easy to relate the observed level of antigen binding to the level of first antibody, and the level of second antibody to that of antigen. This addresses a potential issue in interpreting the results, in that the absence of second antibody binding may result either from interference between the epitopes or from poor binding of the antigen to the first antibody. This cannot be resolved in methods where antigen binding is not monitored directly.

Epitope mapping applications are explicitly supported in Biacore 4000, with methods for both set-up and evaluation of pair-wise mapping experiments (described in detail in the *Biacore 4000 Software Handbook)*. Evaluation is divided into five distinct steps, summarized below. Similar principles can be applied in evaluating pair-wise mapping results from other Biacore systems.

Report points for each evaluation step are set just after the end of the respective injections.

First antibody ranking

This step presents captured levels of the first antibody. The amount of first antibody is fundamental in determining the possible response levels in subsequent steps, and may vary between antibodies. A cut-off boundary can be set to represent the minimum acceptable level: below this level, the amount of captured first antibody is judged to be too low for reliable measurement in subsequent steps, and the antibody pairs represented by these points are excluded from the evaluation.

Antigen ranking

Even with sufficient first antibody captured, binding of antigen may vary, and results for binding of the second antibody will be inconclusive if the level of antigen bound is too low. As for first antibody binding, a cut-off boundary can be set to exclude points with response values below an acceptable limit. The binding data may be shown as relative response values or as percent of expected binding (based on the amount of first antibody captured and the relative sizes of antibody and antigen).

Second antibody ranking

The second antibody ranking displays the binding levels of second antibody, either as relative response or as percent of expected binding (based on the amount of first antibody captured and the relative sizes of antibody and antigen). Here again a cut-off boundary can be set to differentiate between positive and negative binding.

Dissociation fit

An additional component in the evaluation is estimation of the dissociation rate of antibodies classed as positive binders in the second antibody ranking step. This information is not essential for pair-wise mapping itself, although it can be useful in judging the status of borderline results. However, it is often useful in the context of antibody development for immunoassay reagents, and is obtained without additional experimental design or set-up from determinations with Biacore systems.

Result matrix

The results of pair-wise epitope mapping are collected in a matrix of binding values, with first and second antibodies in columns and rows respectively. Cells in the matrix are classed as positive (meaning independent epitopes) or negative (interfering epitopes) on the basis of the cut-off boundary set for the second antibody ranking step. If the cut-off boundaries have been judiciously set in the first antibody and antigen ranking steps, the classification in the

matrix reflects only those pairs where second antibody binding is potentially reliable.

6.2 Peptide inhibition

Epitope mapping by peptide inhibition is based on the principle that a peptide representing a structural epitope will inhibit binding to antigen of an antibody directed against that epitope (Figure 6-2). Inhibition may be partial or complete depending on how well the peptide is structurally congruous with the epitope.

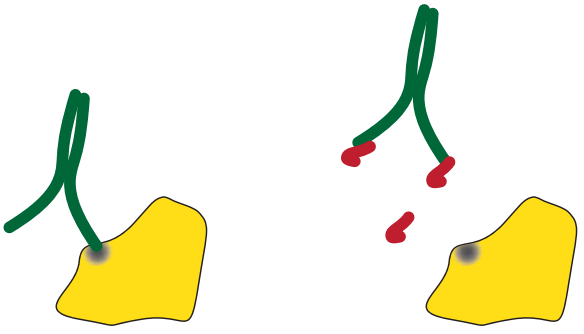


Figure 6-2. Principle of epitope mapping by peptide inhibition principle. In the absence of peptide (left), antibody can bind to the epitope. Added peptide that mimics the epitope binds to the antibody (right) and blocks binding to the antigen.

An assay of this kind can be set up with either the antigen or antibody attached to the surface. Antigen may be attached permanently and regenerated: antibodies are captured to allow investigation of multiple antibodies using the same sensor surface.

7 Troubleshooting assays

This chapter considers the causes and suggested solutions to common problems with Biacore-based assays. The material is divided into 4 sections:

- Ligand attachment
- Analyte binding
- Dealing with non-specific binding
- Unexpected sensorgram shape

7.1 Troubleshooting ligand attachment

This section deals with the problem that the amount of ligand attached to the surface is too low for the experimental purpose.

7.1.1 Covalently attached ligand

A schematic sensorgram for immobilization of a macromolecular ligand on Sensor Chip CM5 is illustrated in Figure 3-3. While details differ according to the chemistry and the specific molecules involved, there are some common features that can help in diagnosing problems with ligand immobilization.

Symptom	Cause	Remedy
Poor response increase during ligand injection	Immobilization pH unsuitable	Optimize immobilization pH
Poor response increase during ligand injection	Too high ionic strength in immobilization buffer	Use low ionic strength (10 mM monovalent cations). Ligands kept in stock solution with physiological ionic strength should be diluted at least 10-fold or otherwise desalted.
Poor response increase during ligand injection	Ligand concentration too low	Test higher concentrations
Ligand binds during injection but is not retained	Ligand lacks accessible target groups for immobilization chemistry	Try a different chemistry or use capturing

Symptom	Cause	Remedy
Ligand binds during injection but is not retained	Buffer components such as Tris or additives such as sodium azide compete with the ligand for immobilization	Make sure the buffer is appropriate for the immobilization chemistry.
Immobilization level low	Old EDC/NHS solutions	Use fresh EDC and NHS solutions.
One or more injections do not show the characteristic response	Solutions wrongly placed in autosampler	Check the ***Rack Positioning*** information in the software for the immobilization procedure.

7.1.2 Captured ligand

Ligand capture by high affinity binding to an immobilized protein involves attachment of the capturing molecule to the sensor surface followed by binding of the ligand to the capturing molecule. From the viewpoint of troubleshooting, the first step is equivalent to immobilizing a ligand, while the second is equivalent to binding analyte.

There are however some specific capture situations supported by ready-to-use sensor chips that can present individual problems in ligand attachment. Laboratory protocols with recommended conditions are available from GE Healthcare.

- Capture of biotinylated ligands on Sensor Chip SA or with the Biotin Capture Kit requires that excess biotinylation reagents are rigorously removed from the ligand solution (suitably be gel filtration on microspin columns). Any residual biotinylation reagents in the solution will compete with ligand for attachment to the surface and reduce capture efficiency. Removal by two cycles of desalting is recommended.
- Capture of histidine-tagged ligands by metal chelation on Sensor Chip NTA requires that buffers are free from chelating agents (EDTA is recommended for ligand removal). The efficiency and stability of capture varies considerably with the number, length and location of the polyhistidine tags on the ligand molecule, but generally improves with reduced ligand levels. If capture on Sensor Chip NTA is unsatisfactory, you may want to try using longer or multiple polyhistidine tags, or capture the ligand on anti-histidine antibodies instead.

7.2 Troubleshooting analyte binding

7.2.1 Analyte capacity too low

Estimate the activity of the attached ligand in terms of the measured analyte binding capacity in relation to the theoretical value (Section 3.2.3). If analyte binding is unacceptably low, the ligand may have lost activity in the low ionic strength, low pH immobilization buffer, been inactivated by the covalent attachment chemistry, or had low activity in the starting material. Try using a different chemistry or switch to a capturing approach.

Occasionally, the attachment chemistry may inactivate the ligand by direct modification of the binding site. In such cases, attaching the ligand in the presence of a reversible binder that blocks the active site can help to preserve activity. It is of course essential that the binder itself is not susceptible to the attachment chemistry. This approach has proved successful in work with some target molecules for low molecular weight analytes.

7.2.2 Analyte binding too high

Analyte binding capacity that exceeds the theoretical maximum value usually indicates either that the binding stoichiometry is more than expected (either because there are multiple binding sites on the ligand or because the analyte is aggregated), or that the analyte is binding indiscriminately to the ligand or the surface matrix. In the former case, it is in principle possible to saturate the surface with sufficiently high analyte concentrations, giving an experimental value for the observed stoichiometry. In the case of indiscriminate binding, the surface can often not be saturated and the binding level continues to increase with analyte concentration even at very high concentrations.

7.3 Dealing with non-specific and unwanted binding

7.3.1 Non-specific binding

Non-specific binding refers to indiscriminate binding of the analyte or other component in the sample to either the ligand or the sensor surface matrix. Non-specific binding can give false positive results in screening assays and incorrect results in concentration assays. In determination of kinetics and affinity, non-specific binding generally gives results that do not fit to an interaction model. Typically, non-specific binding of analyte gives response levels that continue to increase with increasing sample concentration, instead of reaching a saturation plateau.

Binding to the sensor surface matrix is usually revealed by a binding response on the reference surface. Subtraction of the reference response is not sufficient to correct for non-specific binding since it is difficult to establish that binding to

the reference surface and the non-specific component of binding to the active surface are equivalent (high ligand levels can mask dextran binding sites and reduce non-specific binding to the surface matrix). Non-specific binding of this kind can often be reduced by addition of soluble dextran to the samples, to compete for binding to the dextran on the sensor surface. Soluble dextran is available for this purpose from GE Healthcare as NSB Reducer.

Non-specific binding to the ligand does not give a response on a reference surface that lacks ligand but usually shows as non-saturatable binding to the active surface. Binding of non-analyte components may be an issue with complex samples such as cell extracts or serum. General principles for minimizing this kind of binding are to use physiological or higher ionic strength in the sample and running buffer and to include detergent if this does not interfere with the assay. If these measures are not sufficient, choose a different ligand (for example a different clone for monoclonal antibody ligands).

Sometimes the analyte binds promiscuously to the ligand (for example, many low molecular weight compounds bind with low affinity to multiple sites on serum albumin, giving behavior that resembles non-specific binding). In such cases, it may be possible to obtain useful information about high-affinity binding sites from sensorgrams obtained at relatively low sample concentrations, where low affinity binding does not dominate the observed response. Adjustment of buffer conditions for the assay can also help to reduce the effect of promiscuous binding.

7.3.2 Unwanted binding

Unwanted binding refers to the specific binding of non-analyte components to the ligand, or binding of the analyte to non-ligand components such as a capturing molecule on the surface. This kind of interference is best dealt with by careful assay design. For example, avoid antibody subclasses that bind complement factors for measurements in serum, or use Fab fragments that lack the complement-binding sites.

7.4 Unexpected sensorgram shapes

This section considers interpretation and correction of unexpected sensorgram behavior. There will always be individual differences in sensorgram shape according to interaction characteristics, but the following general characteristics are “expected” with due allowance for bulk refractive index effects:

- The baseline should be stable within the analysis cycle. Minor baseline shifts between cycles may be acceptable.

- The response should not decrease progressively during sample injection (with the exception of bulk shifts resulting from refractive index differences).
- The response should not increase progressively during dissociation (with the exception of bulk shifts resulting from refractive index differences).
- Reference-subtracted response overshoot at the start and end of sample injection should be small.
- The response should not go below the baseline level during dissociation.

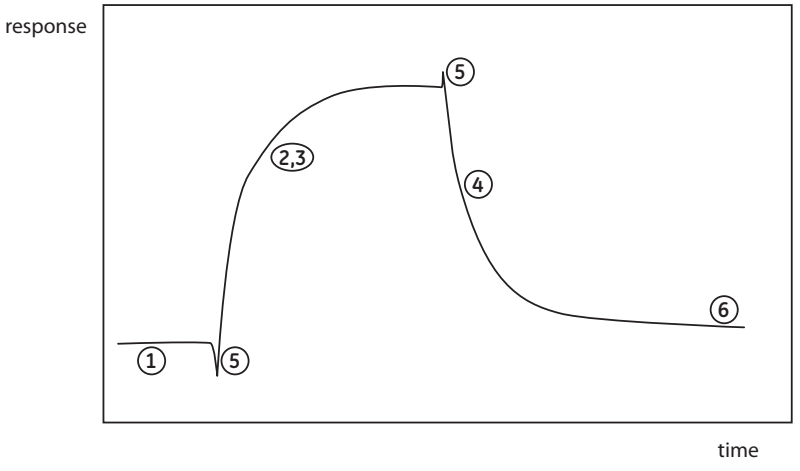


Figure 7-1. Generalized features of the "expected" shape of reference-subtracted sensorgrams.
1: stable baseline.
2: positive response during sample injection.
3: increasing or stable response during injection.
4: decreasing response during dissociation
5: small reference subtraction spikes at the start and end of injection
6: response does not go below baseline

7.4.1 Unstable baseline

Some baseline drift is common in the first few cycles of an assay, and 3 to 5 start-up cycles are recommended to allow the assay to stabilize. Always check the baseline in the reference and active sensorgrams separately: although a drifting baseline may seem to be corrected by reference subtraction, it may indicate inadequate assay optimization and complicate interpretation of the results.

It is relevant to distinguish between baseline drift during an analysis cycle and shifts in baseline response between cycles. The latter is most commonly caused by regeneration characteristics, and can usually be ignored if the surface

binding capacity is not affected (as shown by reproducible binding levels for control samples).

Baseline instability may also be caused by faults in the temperature control system. If this occurs (indicated by a warning on the screen) call your GE Healthcare service representative.

Baseline constantly increasing

- Make sure the running buffer is prepared from pure reagents using clean glassware, and that the instrument flow system is thoroughly cleaned after the previous experiment. This is particularly important for applications using hydrophobic surfaces (Sensor Chip HPA and to a lesser degree Sensor Chip L1).

Baseline constantly decreasing

- If you are using a capturing approach for attaching your ligand, check the stability of the capturing interaction. For work with low molecular weight analytes, you may be able to cross-link the ligand to the capturing molecule after capture to stabilize the surface, but this results in permanent attachment of the ligand. Cross-linking the ligand generally reduces the binding activity for protein analytes.
- Downward drift of the baseline can also be the result of immobilizing a multimeric ligand so that not all subunits are attached to the surface. If the multimer dissociates, subunits will be lost, in most cases resulting in loss of analyte binding capacity as well as baseline response. This behavior can sometimes be prevented by crosslinking the ligand with EDC/NHS after it has been attached to the surface, although the analyte binding activity may be reduced.

7.4.2 Sample response below baseline

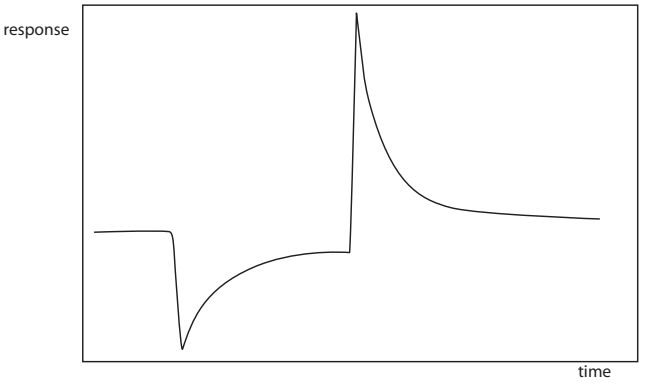


Figure 7-2. Sample response below baseline is usually be caused by a negative bulk refractive index shift.

Responses may fall below baseline during sample injection if the refractive index of the sample is lower than that of running buffer, so that a negative bulk refractive index shift is associated with the sample injection. In such cases, there should be a corresponding positive shift at end of the injection: response levels measured after the end of the injection will still be reliable.

Higher relative response in the reference flow cell than the active flow cell, resulting from sample binding to the reference cell can also cause a shift below baseline in the reference-subtracted sensorgram.

Both of these situations can usually be identified by examining the active and reference sensorgrams separately.

7.4.3 "Humpbacked" sensorgrams

Sensorgrams with a "humpbacked" appearance during sample injection (where the response reaches a maximum and then decreases, see Figure 3-4) are relatively common when the detergent concentration in the running buffer is below the critical micelle concentration (CMC). The recommended concentration of Surfactant P20 is 0.05%. This detergent effect is usually relatively small, and may go unnoticed in experiments with macromolecular interactants where responses are in the range of several hundred RU and above.

There are other possible causes of "humpbacked" sensorgrams, for example initial binding of a large analyte that is then displaced by a slower but tighter binding smaller molecule. You should be aware that this sensorgram appearance does not always indicate a problem with the assay.

7.4.4 Anomalous response during buffer flow

Anomalous responses when only buffer is flowing through the system, including both upward drift and response levels below baseline, may be caused by contamination of the flow system with material from previous runs. Make sure the instrument is maintained as described in the Instrument Handbook. If this behavior is observed, clean the flow system thoroughly as instructed in the Instrument Handbook, then allow the instrument to equilibrate with a low flow of running buffer at least overnight.

7.4.5 Response subtraction spikes

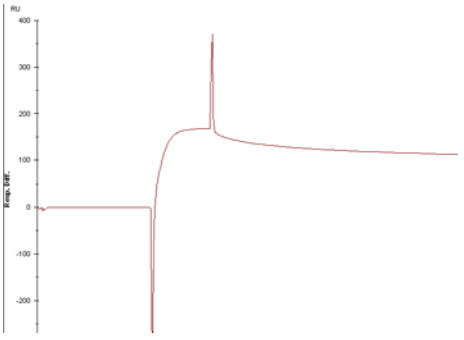


Figure 7-3. Reference subtraction spikes at the beginning and end of a blank (buffer) injection.

Some overshoot at the start and end of the sample injection is normal in reference-subtracted sensorgrams from systems with serially arranged flow cells, particularly at low flow rates. This results from slight misalignment of the reference and active sensorgrams together with a significant bulk refractive index effect (Figure 7-3). This should not affect more than about 1 second of the sensorgram. More serious overshoot may indicate malfunction of the sample injection system, and requires attention from your GE Healthcare service representative.

7.4.6 Regular disturbances

Disturbances (usually small) that occur repeatedly at the same time in each cycle (Figure 7-4) are generally attributable to pump flow-fill operations.

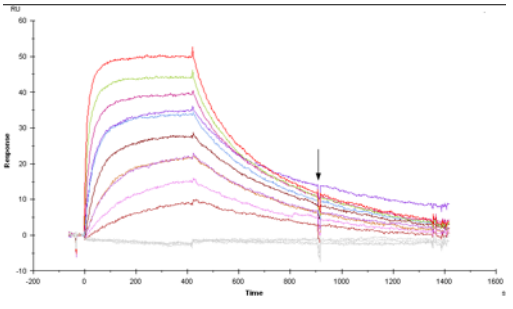


Figure 7-4. Disturbances that occur at the same time in each cycle are attributable to automatic aspects of instrument operation such as pump flow-fill.

Appendix A Analysis of kinetics and concentration measurements

A.1 Basic principles of kinetics and affinity

This section describes the basic principles for kinetics and affinity analysis of 1:1 interactions. Similar principles apply to other interaction models with appropriately modified equations.

A.1.1 Kinetic rate equations

The kinetic evaluation procedure determines association and dissociation constants by fitting the experimental data to a 1:1 interaction model between analyte A and ligand B:

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

where: k_a is the association rate constant ($M^{-1}s^{-1}$)

and k_d is the dissociation rate constant (s^{-1})

The net rate of complex formation during injection is given by

$$\frac{d[AB]}{dt} = k_a[A][B] - k_d[AB]$$

and the rate of dissociation after the end of the injection is

$$\frac{d[AB]}{dt} = -k_d[AB]$$

The concentration of complex formed is measured in RU by the SPR response: if the total ligand concentration $[B]_0$ is also expressed in RU (as the maximum analyte binding capacity), the rate equations can be written in terms of response values instead of concentrations:

$$\frac{dR}{dt} = k_a C R_{max} - (k_a C + k_d)R$$

Because interaction occurs at the surface of the sensor chip, analyte must be transferred laterally in the flow cell from the bulk solution to the surface before interaction with ligand can take place. In a controlled flow system such as Biacore, this transfer is a diffusion-limited process, referred to as *mass transfer*, that can be described by a well-defined mathematical model. The analyte

concentration available for interaction with the ligand is the concentration at the sensor surface $A_{surface}$, which is related to the concentration in the injected sample A_{bulk} by the equation:

$$A_{bulk} \underset{k_m}{\overset{k_m}{\rightleftharpoons}} A_{surface}$$

where k_m is the *mass transfer coefficient*, describing the diffusion-controlled transfer from bulk solution to the surface. The transfer properties are the same both to and from the surface, so that k_m applies in both directions.

The mass transfer coefficient k_m is a function of the flow rate, flow cell dimensions and diffusion properties of the analyte molecule:

$$k_m = 0.98\left(\frac{D}{h}\right)^{2/3}\left(\frac{f}{0.3 \cdot w \cdot l}\right)^{1/3}$$

where D is the diffusion coefficient of the analyte ($m^2 \cdot s^{-1}$)

f is the volume flow rate of solution through the flow cell ($m^3 \cdot s^{-1}$)

h, w, l are the flow cell dimensions (height, width, length in m)

To minimize mass transport limitations in kinetic determinations, experiments should be run at moderate to high flow rates (30 µl/min or more).

The mass transfer coefficient can be adjusted approximately for the molecular weight of the analyte and for the conversion of surface concentration to RU to give a term called the *mass transfer constant* k_t:

$$k_t = k_m \times MW \times 10^9$$

A further modification of this expression gives the *flow rate-independent component* of the mass transfer constant, referred to as ***tc***:

$$t_c = \frac{k_t}{\sqrt[3]{f}}$$

For globular proteins with molecular weight of the order of 50,000 daltons, typical values for ***tc*** are of the order of 10^8 $RU \cdot M^{-1}s^{-1}$. Reported values that differ greatly in order of magnitude (e.g. 10^{12} or 10^{14}) may indicate that the parameter is not significant for the fitting (i.e. that the observed binding is not limited by mass transfer).

Mass transfer is incorporated into the curve-fitting models by modifying the concentration of analyte available for binding to ligand:

A.1.2 Steady-state affinity equations

At steady-state, the net rate of complex formation is zero:

$$\frac{dR}{dt} = k_a C R_{max} - (k_a C + k_d)R = 0$$

so

$$k_a C R_{max} = (k_a C + k_d)R$$

Rearranging and setting $R=R_{eq}$ (the equilibrium response level) gives:

$$R_{eq}\left(\frac{k_a}{k_d}C + 1\right) = \frac{k_a}{k_d}CR_{max}$$

Setting $k_a/k_d=K_A$ (the equilibrium association constant) gives

$$R_{eq} = \frac{K_A C R_{max}}{K_A C + 1}$$

The value of K_A is obtained by fitting a plot of R_{eq} against C to this equation. K_D is calculated as the inverse of K_A ($K_D=1/K_A$).

A.1.3 Fitting procedure

The fitting procedure used to determine rate constants from experimental data uses numerical integration methods with an iterative approximation algorithm to find the best solution to the equations given above. The closeness of fit is judged in terms of the chi-square value which describes the deviation between the experimental and fitted curves:

$$\text{chi-square} = \frac{\sum_{1}^{n} (r_f - r_x)^2}{n - p}$$

where r_f is the fitted value at a given point

r_x is the experimental value at the same point

n is the number of data points

and p is the number of fitted parameters.

The fitting algorithm seeks to minimize chi-square, and is judged to be complete when the difference in chi-square values between successive iterations is sufficiently small.

A.1.4 Assessing the fit

Kinetics

The rate constants reported by kinetic evaluation are determined in terms of a 1:1 interaction model, and it is important to realize that they are only valid in that context. If the interaction mechanism is not a simple 1:1 binding, the fitted curves will deviate to some extent from the experimental data and the reported constants will not be a true representation of the interaction kinetics. The apparent 1:1 binding constants can still be used for comparative studies of observed binding rates, but it is important in reporting the values to emphasize that they are empirical and not mechanistic constants.

There are two major tools for assessing the significance of the reported constants: the closeness of fit between the fitted and experimental curves and the statistical significance of the parameters.

Closeness of fit

Visual inspection of the residual plot or of the fitted curves overlaid on the experimental data gives an indication of the closeness of fit. Ideally, the residuals will scatter randomly around zero over a range that corresponds to the short-term noise in the detection system (approximately ±1 to 2 RU). Deviations from ideal fitting appear as systematic variations in the residuals, imparting a non-linear shape to the residual plot. Judge the residual range and shape in proportion to the response ranges in the experimental sensorgrams and in relation the goal of the investigation.

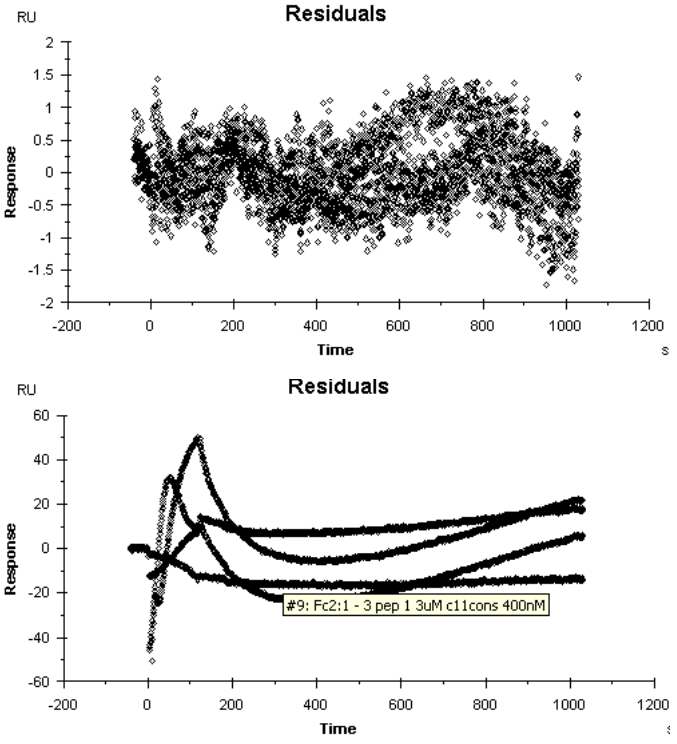


Figure A-1. *Residual plots from a good fit (top) and a poor fit (bottom). Note the different response scales in the two plots (top ±2 RU, bottom ±60 RU).*

The chi-square value is a quantitative measure of the closeness of fit, and in an ideal situation will approximate to the square of the short-term noise level. It is however difficult to recommend absolute values for acceptance limits for chi-square: the values need to be considered from case to case in combination with assessment of the shape of the residuals (chi-square is related to the overall range of the residuals but is not affected by the shape of the residual curve).

Significance of parameters

Fitting experimental data to a mathematical interaction model will return values for all parameters in the model, regardless of whether they are significant or not. As an example, sensorgrams that are completely limited by mass transfer will give values for the kinetic rate constants even though the experimental data does not contain any kinetic information.

The significance of the parameter values returned by the fitting procedure is indicated by the ***standard error*** or ***T-value***, which broadly speaking represents the degree to which the value can be varied without significantly affecting the closeness of fit. The T-value is the standard error divided by the parameter value. A high standard error or low T-value indicates that the fitting is insensitive

to changes in the parameter value: in other words, the value returned for the parameter has little significance.

It is important to examine the significance of values calculated by the software, to avoid the trap of reporting "constants" that have no significance. In the example of completely mass transfer limited interactions, the rate constants will have a high standard error (low T-value), since it does not matter for the fitting result what value will be returned.

Note: *Even if the significance of a parameter is low, so that any value within a wide range will give the same fit to the experimental data, the fitting algorithm will always return the same value from the same starting data. Consistency of returned values is not an indication of significance.*

For the 1:1 binding model, an additional indicator of the parameter significance is the ***U-value***. This is a parameter that represents the uniqueness of the calculated rate constants and R_{max}, determined by testing the dependence of fitting on correlated variations between selected variables. Lower values indicate greater confidence in the results. A high value (above about 25) indicates that the reported kinetic constants contain no useful information.

Affinity

To obtain a robust fit to a plot of R_{eq} against C for affinity determination, it is important that the range of analyte concentration is wide enough to reveal the curvature of the plot in full. In particular, if the highest concentration is too low in relation to the calculated K_D value, the reported values will be uncertain even if the fit is apparently good and the chi-square value is low.

As a general guideline, the fit will be reliable if the highest analyte concentration is at least twice the calculated K_D value. If the highest analyte concentration is less than $2 \times K_D$, you should treat the reported K_D with caution.

A.2 Interaction models for kinetics

This section gives a brief description of the standard kinetic interaction models provided with Biacore systems.

A.2.1 1:1 binding

This interaction model describes one molecule of analyte A binding to one molecule of ligand B:

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

where k_a is the association rate constant

and k_d is the dissociation rate constant

A.2.2 1:1 dissociation

This model fits the dissociation phase only to an exponential model. The dissociation rate is independent of the analyte concentration in the injected sample, so that dissociation rate constants can be obtained for samples with unknown concentration.

This model does not include a mass transfer coefficient. If mass transfer limitations are significant, dissociated analyte will be washed away from the surface with reduced efficiency and the dissociation rate constant will be underestimated.

A.2.3 Bivalent analyte binding

The bivalent analyte binding model describes the interaction of monovalent ligand with analyte molecules that carry two identical and independent binding sites.

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

$$AB + B \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} AB_2$$

where k_{a1} and k_{d1} are the association and dissociation rate constants for the first site

and k_{a2} and k_{d2} are the association and dissociation rate constants for the second site

Once binding has occurred at the first analyte site, binding at the second site is facilitated by the proximity of analyte and ligand. Similarly, analyte molecules that are attached at both sites are not released from the surface until dissociation occurs at both sites, so the observed dissociation rate is much slower than that seen for a single site. The resulting enhanced binding is often called **avidity** to distinguish it from single site binding affinity.

Note: *The association rate constant for interaction at the second site is reported in units of $RU^{-1}s^{-1}$. This is because both the interacting components are present on the surface and are measured in RU, not in true concentrations.*

A.2.4 Heterogeneous ligand

This model describes interaction of one analyte with two independent ligands (or ligand sites) on the surface. The observed binding is the sum of the interaction with the two ligands.

$$A + B1 \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} AB1$$

$$A + B2 \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} AB2$$

where k_{a1} and k_{d1} are the association and dissociation rate constants for one ligand

and k_{a2} and k_{d2} are the association and dissociation rate constants for the other ligand

A.2.5 Interaction models for affinity

1:1 steady state binding

The ***Affinity*** evaluation item described in Chapter 12 fits a plot of response against concentration to the equation (Section A.1.2)

$$R_{eq} = \frac{K_A C R_{max}}{K_A C + 1} + \text{offset}$$

where ***offset*** is a constant term that gives the intercept of the fitted curve on the y-axis. Without this term the fitted curve is forced to go through the origin of the plot.

The parameters $\boldsymbol{K_A}$, $\boldsymbol{R_{max}}$ and ***offset*** are fitted as variables. The affinity is reported as a K_D value, which is the inverse of K_A. This function gives reliable fitting if the highest analyte concentration used is at least twice the K_D value.

A.3 Concentration measurements

A.3.1 Calibration curve fitting

Calibration curves for concentration measurement are fitted to either linear or 4-parameter equations. Custom equations for calibration curves can be defined in some Biacore systems.

For linear fitting, the points are fitted to the equation

$$y = \text{slope} * x + \text{intercept}$$

The equation for a 4-parameter fit is

$$y = R_{hi} - \frac{R_{hi} - R_{lo}}{1 + \left(\frac{x}{A_1}\right)^{A_2}}$$

where y and x are the plot coordinates

R_{hi} and R_{lo} are fitting parameters that correspond to the maximum and minimum response levels respectively

A_1 and A_2 are additional fitting parameters

The closeness of fit is reported for linear fitting as the coefficient of determination R^2 and for 4-parameter fitting as the chi-square value.

A.3.2 Calibration trends

Some Biacore systems support *calibration trends* to compensate for drift in the calibration throughout the course of the assay. With this approach, calibration curves are measured at the start and end of the assay and at intervals, and an individual calibration curve is constructed for each sample analysis cycle by interpolating between the measured calibration curves.

A.3.3 Calibration-free concentration analysis

Evaluation of calibration-free concentration relies on fitting the data to a model for 1:1 interaction (see Section A.1) where the mass transfer coefficient is provided (through calculation from the diffusion coefficient and flow cell characteristics) and the analyte concentration is evaluated as a global variable. This model is equivalent in terms of the interaction description to the model for evaluation of 1:1 kinetics.

Appendix B Solvent correction principles and practice

B.1 Introduction

Solvent correction refers to the procedures used to adjust measured responses for the effects of varying concentrations of organic solvents. These procedures are relevant primarily for work with small organic analytes that require inclusion of organic solvents (commonly dimethyl sulfoxide, DMSO) in the buffer to maintain analyte solubility.

Simple subtraction of a reference response is not sufficiently accurate for these purposes when differences in bulk shifts between samples can be of the same order of magnitude as (or larger than) the expected response from analyte binding. One reason for this is that the volume of solution accessible to sample will differ on the reference and active surfaces unless the two surfaces have the same attached protein concentration. As a result of the volume excluded by ligand molecules, the bulk shift will be smaller on the active surface than the reference surface (Figure B-1).

There are other factors in addition to excluded volume involved in solvent correction that are outside the scope of this discussion, so that solvent correction is essentially an empirical procedure.

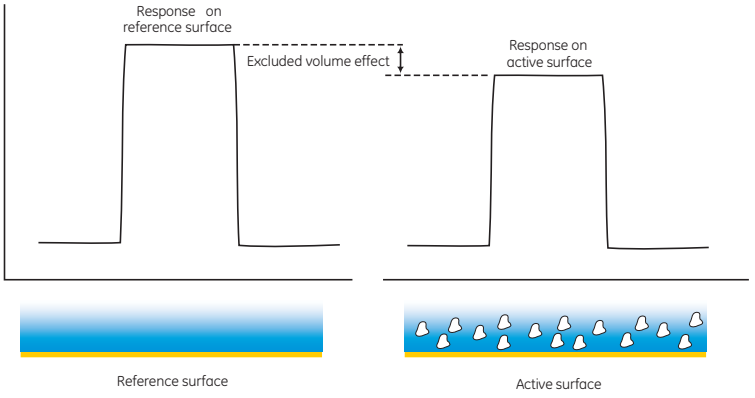


Figure B-1. Bulk solution is excluded from the volume occupied by ligand molecules on the active surface, so the contribution of bulk solution to the relative response is smaller than on the reference surface.

B.2 Requirement for solvent correction

Solvent correction is necessary under a combination of three circumstances, commonly met in work with low molecular weight analytes such as drug candidates:

- the expected analyte responses are low,
- the ligand is a macromolecule immobilized at a high density on the surface,
- the bulk response is high in relation to the measured binding response.

In drug discovery and development work, the analytes are often small molecules which give correspondingly low response values (typically of the order of 10-50 RU or less). High levels of immobilized ligands (several thousand RU) are used to maximize the analyte response, enhancing the excluded volume effect described above. Samples generally include DMSO to maintain solubility, resulting in high bulk responses. A difference of 1% (percentage points) in DMSO concentration corresponds to a difference of about 1200 RU in response, so that small variations in DMSO concentration, unavoidable in the preparation of diverse samples, easily lead to variations in bulk response of the same order of magnitude as the expected sample responses.

B.3 Solvent correction principles

Solvent correction values are determined by injecting blank samples containing a range of DMSO concentrations and plotting the reference-subtracted response from the active surface against the relative response from the reference surface. This creates a calibration curve, referred to as the solvent correction curve, for the solvent effect as a function of the reference response. Each sample measurement can then be adjusted for the solvent effect on the basis of the response on the reference surface (see Figure B-2).

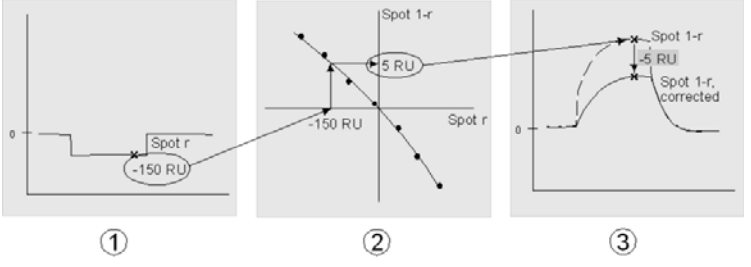


Figure B-2. The principle of solvent correction. **1.** The sensorgram from the reference surface shows a bulk displacement (-150 RU in the illustration) during sample injection because the sample and running buffer are not exactly matched. **2.** From the solvent correction curve, a displacement of –150 RU in the reference sensorgram corresponds to a solvent effect of +5 RU in the reference-subtracted sensorgram. **3.** The reference-subtracted sensorgram is corrected for the solvent error. This procedure is applied to every point during sample injection.

Solvent correction is applied only to response levels during sample injection, since the correction adjusts for differences in bulk response between different samples.

B.4 Preparing solutions for solvent correction

It is important to match samples and running buffer as closely as possible with respect to refractive index to minimize bulk refractive index effects, so that solvent correction values are kept small. It is particularly important to strive for exactly the same DMSO concentration in samples and running buffer and to minimize variations in DMSO content between samples. Bear the following in mind when preparing samples for assays where solvent correction is necessary:

- Use carefully designed buffer preparation and sample dilution protocols to ensure consistent DMSO concentrations. Samples that are stored in DMSO should be diluted in such a way as to give the same final DMSO concentration as in the running buffer.
- Cover the sample plates with foil directly after preparation to avoid changes in DMSO concentration resulting from evaporation and absorption of water from the air. Remember that DMSO is hygroscopic.
- If you are using a laboratory robot to prepare samples, consider running a test with blank samples to establish the range of variability of DMSO concentrations in the samples. Run solvent correction cycles that cover the expected range of variability.

Laboratory protocols for preparation of samples and solvent correction solutions may be found on www.gelifesciences.com/biacore or obtained from GE Healthcare.

B.5 Assessing solvent correction procedures

Solvent correction curves often show a negative slope with a slight curvature (Figure B-3, left panel), but the slope and shape is not predictable and curves with positive slope or even with a maximum or minimum within the curve may be acceptable.

It is however important that the solvent correction curves fit the experimental points closely. Poor fitting of the curves to the experimental points (Figure B-3, center panel) can introduce uncertainties in the solvent correction factors that are the same order of magnitude as the factors themselves, potentially introducing additional errors in the sample measurements.

Sample responses that lie outside the range of the solvent correction curves (Figure B-3, right panel) cannot be corrected and will be omitted from evaluation of solvent corrected data. Solvent correction curves may be extrapolated in the software, but this option should only be used for small extensions of the solvent correction range (less than 10% of the measured range) since the curve shape is not predictable.

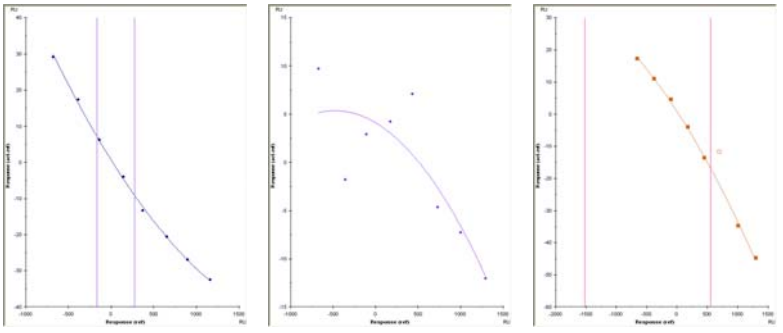


Figure B-3. Some examples of solvent correction curves.
Left: Acceptable curve, with close fitting to the experimental points and covering the sample response range well.
Center: The experimental points scatter widely around the fitted solvent correction curve. Badly fitted curves should be treated with caution.
Right: The solvent correction curve does not cover the whole sample response range. Sample responses outside the range of the curve will not be corrected.

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