Biacore

Concentration Analysis Handbook
Contents

1 Introduction
   1.1 Principles of Biacore measurements .......................................... 5
   1.2 Biacore in concentration measurement .................................... 5
   1.3 Why use Biacore? ........................................................................... 6
   1.4 Assay development overview ...................................................... 7

2 Terminology
   2.1 Terminology for Biacore-based assays ...................................... 9
   2.2 Performance criteria ................................................................. 11
      2.2.1 Specificity, selectivity and cross-reactivity............................... 11
      2.2.2 Accuracy and recovery.......................................................... 12
      2.2.3 Precision ........................................................................... 12
      2.2.4 Limit of detection (LOD)......................................................... 14
      2.2.5 Limits of quantitation (LOQ)................................................... 14
      2.2.6 Linearity ............................................................................. 16
      2.2.7 Range................................................................................ 16
      2.2.8 Robustness ..................................................................... 17
      2.2.9 Sensitivity ................................................................. 17

3 Assay formats
   3.1 Calibrated direct binding assays .............................................. 20
      3.1.1 Single step response level measurement .................................. 21
      3.1.2 Binding rate measurements ................................................... 22
      3.1.3 Sandwich methods ............................................................. 22
   3.2 Calibrated indirect assays .......................................................... 24
      3.2.1 Inhibition assays ................................................................. 24
      3.2.2 Surface competition ............................................................ 25
   3.3 Calibration-free concentration assays .................................... 26
      3.3.1 Scope and limitations of calibration-free assays ................ 26
      3.3.2 Setting up calibration-free assays ...................................... 27
   3.4 Choice of assay technique .......................................................... 28
   3.5 Choice of ligand and other reagents ........................................ 29
      3.5.1 Practical approaches to reagent selection............................. 29
      3.5.2 Direct binding assays .......................................................... 29
      3.5.3 Inhibition assays ................................................................. 31
      3.5.4 Surface competition assays ................................................. 31

4 Surface preparation principles
   4.1 Sensor surface properties ......................................................... 33
   4.2 Ligand immobilization methods ............................................... 34
      4.2.1 Amine coupling ................................................................. 34
      4.2.2 Thiol coupling ................................................................. 35
      4.2.3 Aldehyde coupling ......................................................... 36
      4.2.4 Streptavidin-biotin capture ............................................... 37
4.2.5 General capture methods ................................................................. 37
4.2.6 Immobilizing small molecules ....................................................... 38
4.3 Conditions for ligand immobilization .................................................. 38
  4.3.1 Buffer conditions and pre-concentration ....................................... 39
4.4 Strategy for surface preparation ........................................................ 40
  4.4.1 Amount of immobilized ligand ...................................................... 40
  4.4.2 Choice of immobilization method .................................................. 41

5 Surface preparation in practice
  5.1 Conditions for immobilization ......................................................... 43
  5.2 Immobilization procedure .............................................................. 45
    5.2.1 Preparing solutions ................................................................. 45
    5.2.2 Amine coupling ....................................................................... 45
    5.2.3 Surface thiol coupling .............................................................. 46
    5.2.4 Ligand thiol coupling ............................................................... 47
    5.2.5 Aldehyde coupling ................................................................. 48
    5.2.6 Results of immobilization ....................................................... 49
  5.3 Testing the surface .................................................................... 49
  5.4 Troubleshooting surface preparation ............................................. 50
    5.4.1 Low immobilization levels ....................................................... 50
    5.4.2 Low analyte responses ............................................................ 52

6 Regeneration
  6.1 Regeneration scouting strategy ....................................................... 53
    6.1.1 Choice of regeneration solution .............................................. 53
    6.1.2 Testing regeneration conditions ............................................... 54
  6.2 Interpreting regeneration scouting ................................................. 55
    6.2.1 Report point placing ............................................................... 55
    6.2.2 Trend plots .............................................................................. 55

7 Developing and running concentration assays
  7.1 Assay requirement specification ...................................................... 59
  7.2 General practical considerations .................................................... 60
    7.2.1 Preparing samples ................................................................... 60
    7.2.2 Report point placing ............................................................... 60
  7.3 Adjusting the operating range ......................................................... 62
    7.3.1 Direct binding assays ............................................................... 62
    7.3.2 Inhibition assays ..................................................................... 64
    7.3.3 Calibration-free concentration assays .................................... 66
  7.4 Unwanted binding of analyte or detecting molecule ...................... 67
    7.4.1 In direct binding assays ........................................................... 67
    7.4.2 In inhibition and surface competition assays ............................ 67
    7.4.3 In calibration-free assays ........................................................ 68
    7.4.4 Testing for unwanted binding ................................................ 68
    7.4.5 Dealing with unwanted binding .............................................. 69
  7.5 Specificity and cross-reactivity ....................................................... 69
    7.5.1 Measuring specificity .............................................................. 69
1 Introduction

1.1 Principles of Biacore measurements

Biacore™ systems from GE Healthcare exploit the phenomenon of surface plasmon resonance (SPR) to monitor the interaction between molecules in real time. The technology, which is described in more detail in Appendix A, involves attachment of one interacting partner to the surface of a sensor chip, and then passing sample containing other (potential) interaction partners over the surface. Binding of molecules to the sensor surface generates a response which is proportional to the bound mass, and changes in amount bound can be detected down to a few picograms per square millimeter on the sensor surface. Binding events are followed in real time and a range of interaction characteristics can be determined. Among the questions that can be addressed with Biacore are:

- The specificity of biomolecular interactions, investigated by testing the extent of binding between different pairs of molecules.
- The kinetics and affinity of an interaction, investigated by analyzing the time curve and level of binding in terms of molecular interaction models.
- The concentration of specific molecules present in the sample, determined from the response or rate of binding obtained from the sample.

1.2 Biacore in concentration measurement

The use of Biacore to measure concentration can provide advantages of speed, automation, simplicity and selectivity in comparison with more established techniques. In addition, the selectivity of a Biacore assay is determined primarily by the choice of interacting partner attached to the sensor surface, so that assays can be tailored to specific purposes in a way that may not be possible with other techniques.

Determination of biologically relevant concentrations is fundamental to many fields in both academic and industrial research, and is gaining importance in the field of quality control in pharmaceutical development and production. While any Biacore system can in principle be used to measure concentration, some Biacore systems are designed specifically for concentration assays in GxP environments (covering Good Laboratory Practice GLP, Good Manufacturing Practice GMP and Good Clinical Practice GCP), where demands for relevant and reliable concentration measurement are augmented with demands for secure data handling and documentation.
1 Introduction
1.3 Why use Biacore?

This handbook provides guidelines for how to develop and use concentration assays with Biacore. The information is given as far as possible in general terms without direct reference to specific Biacore systems.

1.3 Why use Biacore?

There are many well-established ways of measuring concentration. What advantages can Biacore-based assays provide over conventional interaction-based methods?

The principles of concentration measurement with Biacore are largely similar to established interaction methods such as ELISA, except that in Biacore the extent of interaction is measured directly, allowing rate-based as well as end-point measurements. Another major difference between Biacore and other methods lies in the real-time, label-free aspects of the measurement. A Biacore-based assay continuously monitors each binding step in the assay procedure, in contrast to many other techniques that only measure the end-point level of the final interactant. A direct comparison of antibody-based assays in Biacore and in an ELISA format reveals a number of advantages with the former:

- Assay procedures for ELISA involve a number of washing steps, where analyte that dissociates from the detecting molecule can be lost. Biacore detects the analyte directly. Because of this, only high-affinity antibodies are suitable for ELISA, whereas Biacore makes less stringent demands on the properties of the detecting molecule.

- ELISA assays require additional steps using secondary reagents to measure the amount of antibody-antigen complex formed on the ELISA plate. Biacore measures the amount directly and requires no additional reagents, although additional reagents may be exploited to enhance the sensitivity and/or specificity of the assay.

- The result of an ELISA assay is only seen after the final step. Biacore monitors each step in the process, providing quality control of the assay procedure even if only a single time point is actually used for the concentration determination.

Another advantage inherent in the detection method is that the measurements are non-invasive. Although the detection technology is optical in character, the light does not actually penetrate the sample (see Appendix A). In consequence, there is no interference from absorption by colored samples or light scattering by turbid samples. Even samples such as whole blood and milk can be analyzed with the same confidence as clear and colorless solutions.
1.4 Assay development overview

Setting up a concentration assay on a Biacore system involves the following main steps:

1. Select an assay format, including choice of interacting partner(s) to be used in the assay.
2. Prepare the sensor surface for the assay.
3. Establish conditions for regeneration of the surface to enable repeated analyses on the same surface.
4. Develop the assay conditions using samples containing known analyte concentrations.
5. Validate the assay if required by quantitating the relevant performance parameters according to accepted company or external guidelines.

The process of assay development is considered in Chapters 4-7 in this handbook. Practical aspects of assay validation are discussed in Chapter 8.
1 Introduction
1.4 Assay development overview
2 Terminology

This chapter presents the terminology that is used in the context of concentration measurements with Biacore. Section 2.1 presents terms specific to Biacore-based applications, while Section 2.2 defines the terms used to describe assay performance in the context of validated analytical procedures.

2.1 Terminology for Biacore-based assays

Biacore monitors 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 concentration measurement (see Figure 2-1):

- The partner attached to the surface is called the ligand. Attachment may be covalent or through high affinity binding to another molecule which is in turn covalently attached to the surface (see Chapter 3). In the latter case the molecule attached to the surface is referred to as the capturing molecule. (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 analyte is the molecule for which the concentration is to be measured. In direct binding assays, the analyte binds directly to the ligand. In inhibition assays, the concentration of analyte is measured indirectly through binding of an additional molecule.

- The detecting molecule is the molecule responsible for detecting the analyte. In direct binding assays, the detecting molecule is the same as the ligand. In inhibition assays, the detecting molecule is the molecule that binds to the ligand and generates a response which is blocked by the presence of analyte (see Figure 3-1).

![Figure 2-1. Ligand, analyte and capturing molecule in relation to the sensor surface.](image-url)
2 Terminology
2.1 Terminology for Biacore-based assays

- **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.

- Response is measured in **resonance units (RU)**. The response is directly proportional to the mass concentration of biomolecules on the surface.

- A **sensorgram** is a plot of response against time (see Figure 2-2), showing the progress of the interaction. This curve is displayed directly on the computer screen during the course of an analysis. Sensorgrams may be analyzed to provide information on the rates of the interaction.

- 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 2-2: Schematic illustration of a sensorgram. The bars below the sensorgram curve indicate the solutions that pass over the sensor surface.](image)

- The **sample matrix** is the solution environment in which analyte is present in samples. This may be a simple well-defined solution such as a buffer, or a complex mixture such as a body fluid, cell extract or product formulation. (The term *matrix* is also used to refer to the carboxymethyl dextran on the surface of the sensor chip in Biacore, as described in Section 4.1. This usage is in no way connected to the term sample matrix.)
2.2 Performance criteria

However concentration is measured, there is a set of standardized criteria that measure the performance of the assay within and between assay occasions. Documented performance is desirable in all concentration measurements, and is a requirement for validated assays used in quality control and other contexts. The standard performance criteria defined in the following sections are based on the ICH recommendations¹. Considerations of how to measure performance criteria for a given assay are considered in Chapter 8.

2.2.1 Specificity, selectivity and cross-reactivity

The specificity of an assay is the ability to measure the concentration of analyte without interference from other components that might be present in the sample. Components that may interfere with the assay are typically impurities, degradation products or other matrix components. In the context of an interaction-based assay designed to measure functionally active analyte, inactive analyte molecules may also be considered as potential sources of interference.

Although the terms specificity and selectivity are sometimes used interchangeably they strictly have slightly different meanings. Specificity refers to the ability to measure a single analyte species to the exclusion of others, while selectivity refers to the ability to measure a class of analyte species without distinguishing the individual members of the class. In these terms, for example, an assay that is specific for folic acid will not detect the related molecule tetrahydrofolic acid, whereas an assay that is selective for folic acid derivatives may detect both. Narrow specificity in an assay is not necessarily a requirement: on the contrary, a broad selectivity is desirable for assays intended to measure classes of analytes.

Cross-reactivity is a quantitative measure of specificity and selectivity, and is expressed formally in terms of the ratio of the affinities of different analytes for the ligand or detecting molecule (Figure 2-3). In practice, cross-reactivity in an inhibition assay may be determined from the IC₅₀ values (see Section 2.2.7) for the analytes. In a direct assay, the observed response depends on the molecular weight of the analyte (see Appendix A), and comparisons of B₅₀ values must be made in terms of response divided by molecular weight. A compound that requires 100 times higher concentration to give the same molecular weight-corrected response as the B₅₀ value for the analyte is said to show a cross-reactivity of 1%.

2 Terminology

2.2 Performance criteria

Figure 2-3. Cross-reactivity in a direct assay is determined from calibration curves of molecular weight-corrected response against concentration. In this illustration, compound B shows 1% cross-reactivity with compound A.

2.2.2 Accuracy and recovery

The **accuracy** of an assay describes how well the measured concentrations agree with accepted reference values. The accuracy is determined from measurements made on standard samples in comparison either with the results of an independent reference assay or with quoted values for the standard. In this respect, it is relevant to remember that Biacore-based assays only measure analyte that is capable of binding to the ligand or detecting molecule: the same applies to all interaction-based assays but not to measurements based on e.g. determination of protein content. Discrepancies between results from Biacore and reference values may be observed if the reference assay is not based on the same kind of interaction as the Biacore-based assay. The significance of such discrepancies for acceptance of the assay performance must be assessed for each individual case.

**Recovery** is a term used in relation to the quantitation of accuracy, and refers to the correlation between the measured and expected amounts of analyte in samples spiked with known amounts.

2.2.3 Precision

The **precision** of an assay describes the agreement (degree of scatter) between results obtained from multiple measurements on the same homogeneous sample. Precision may be determined at three levels:

- **Repeatability** is the precision of the assay under the same operating conditions with the same sample over a short period of time (typically replicate measurements within the same experiment, also referred to as intra-assay precision).

- **Intermediate precision** is the precision within the same laboratory over different occasions, different operators, different individual assay
Terminology

- **Reproducibility** is the precision between different laboratories (usually applied to collaborative studies in the standardization of methodology). Intermediate precision and reproducibility are two different aspects of inter-assay precision.

The precision of an assay is usually expressed in terms of the variance, standard deviation (SD) or coefficient of variation (CV) within the series of measurements. For a set of replicate measurements, the standard deviation is given by

\[
SD = \sqrt{\frac{1}{n-1} \sum (y_i - \bar{y})^2}
\]

where \(n\) = number of measurements
and \(y\) = response for a given measurement.

The coefficient of variation is given by

\[
CV(\%) = \frac{SD}{\text{mean}} \times 100
\]

Statistical parameters for variation in measured results may be related to either dose or response values: this is perhaps most common in citations of CV values where the distinction is made between \(CV_{dose}\) and \(CV_{response}\) (Figure 2-4). The \(CV_{response}\) value reflects the consistency of response values for a given concentration, while \(CV_{dose}\) reflects the confidence with which a given response value can be related to analyte concentration. In general, \(CV_{dose}\) values are high at the top and bottom of the measurement range (at the bottom because low responses are difficult to determine accurately, at the top because the standard curve flattens out at high concentrations), and are lowest in the middle of the range. \(CV_{response}\) values, on the other hand, are frequently low even at the top of the dynamic range. \(CV_{dose}\) is in general a better criterion of assay performance than \(CV_{response}\).
Accuracy and precision are frequently confused in common parlance, although they are clearly distinguished in their formal definitions. An assay that is precise but not accurate will always give the same (wrong) answer, while one that is accurate but not precise will give an approximate but correct answer.

### 2.2.4 Limit of detection (LOD)

The limit of detection (LOD) is the lowest analyte concentration that can be detected but not necessarily quantitated as an exact value (see Figure 2-5). For direct assays, the LOD is primarily a function of the signal-to-noise ratio in the measurement itself, and is set in relation to statistical variations in response values for blank samples. A commonly used value is $3 \times \text{SD}$, where SD is the standard deviation of replicate measurements on blank samples.

If the LOD is determined from measurements on blank samples (or from other means of measuring the noise level in the assay when no analyte is present), this parameter does not incorporate any factor relating to experimental variations in source, composition or preparation of samples. These aspects are included in the limit of quantitation, as described below.

### 2.2.5 Limits of quantitation (LOQ)

Determination of the LOQ of an assay requires that the precision and accuracy of the assay are measured over a range of analyte concentrations, in order to determine the concentrations above and below which the performance is acceptable (see Figure 2-5). These concentrations are then the LOQs: different values may be applicable according to whether precision is determined as intermediate precision or reproducibility. (The LOQ should not be based on
repeatability or intra-assay precision, since the value has little meaning in reference to a single assay occasion).

The lower limit of quantitation (LLOQ) is the lowest analyte concentration that can be measured with suitable precision and accuracy. The level corresponding to “suitable” is set according to the purpose and requirements of the assay.

The upper limit of quantitation (ULOQ) is the highest analyte concentration that can be measured with suitable precision and accuracy. There is no upper limit for assay procedures that do not impose restrictions on dilution factors involved in sample preparation: in some cases, however, sample preparation procedures may define a maximum permitted dilution, so that the assay will have an upper limit of quantitation.

Rigorous determination of the LOQ requires extensive measurements over a period of time and by different operators. Potential variation in other equipment such as pipettes, balances and volumetric flasks used in sample preparation as well as batch variation in reagents should also be taken into account. If the demands on documented assay performance are less stringent, a value of $10 \times SD$ (i.e. $3.3 \times LOD$), where SD is the standard deviation of replicate measurements on blank samples, may be used as an initial estimate of the LLOQ. This value can then be verified by using a relatively small number of measurements on samples containing analyte. The unverified initial estimate should however never be quoted as a value for the LOQ.

**Figure 2-5.** Limits of detection and quantitation.
2.2.6 Linearity

The linearity of an assay refers to the ability of the assay to obtain response values that are related to the analyte concentration by a defined mathematical function. Ideally, the function should be linear, if necessary after an appropriate mathematical transformation of the data. For many interaction-based assays, however, a linear relationship cannot be obtained even after mathematical transformation, and in such cases it is acceptable that the relationship between response and analyte concentration is defined by an appropriate mathematical function. Evaluation of concentration measurements in Biacore offers a fully defined four-parameter equation for fitting a curve to the calibration data points.

In quantitative terms, linearity is expressed as the regression coefficient for fitting the data points to a straight line. This approach cannot be used in cases where a linear function is not available. An alternative is to plot the measured analyte concentrations against the expected (known) concentrations and determine the regression coefficient for fitting these points to a straight line. This plot of measured against expected concentrations should always be a straight line regardless of the shape of the function describing response against concentration.

2.2.7 Range

The range of an assay is the interval between (and including) the upper and lower limits of quantitation, i.e. the range within which the precision, accuracy and linearity are acceptable.

A parameter often quoted in relation to the range is the analyte concentration that gives 50% of the maximum response (B50 for a direct binding assay, IC50 for an inhibition assay, see Figure 2-6). Note that this is not necessarily the mid-point of the concentration range.

Figure 2-6. B50 (direct assays, left panel) and IC50 (inhibition assays, right panel) are the analyte concentration that gives 50% of the maximum response.
2.2.8 Robustness

The robustness of an assay is a measure of its capacity to remain unaffected by variations in method parameters. Robustness is related to intermediate precision or ruggedness. While intermediate precision refers to the effect of unintentional variations between assay occasions, operator etc (see Section 2.2.3), robustness is determined by means of deliberate variations in chosen assay parameters. An assay that is robust with respect to all essential parameters will also have a high level of intermediate precision.

2.2.9 Sensitivity

The sensitivity of an assay is not included among the recommended performance criteria for validation, but is worthy of a definition here because the term is often used in several different and to some extent conflicting senses. Formally, sensitivity is defined as the assay response per unit analyte concentration (e.g. RU per µg/ml or RU/µM). This is the slope of the standard curve for the assay (Figure 2-7). For assays where the standard curve is not linear, the sensitivity varies with the analyte concentration. The term “sensitivity” is however sometimes used as a synonym for LOD or LOQ (the lowest concentration that can be detected or measured) or resolution (the smallest difference in concentration that can be determined with confidence). Usage of the term in the formally defined sense (assay response per unit analyte concentration) is recommended to avoid confusion.

Figure 2-7. Sensitivity is the response per unit analyte concentration, which is the slope of the calibration curve. The sensitivity varies over the range of the assay if the calibration curve is not linear.
2  Terminology
2.2  Performance criteria
3 Assay formats

Biacore supports a number of different approaches for measuring concentration, appropriate for different situations. All rely on the specific interaction of the molecule being measured (the analyte) with a detecting molecule.

Assays using calibration curves

- Direct binding assays measure analyte bound directly to the ligand on the sensor surface, either as the response after a fixed time of sample injection or as the initial binding rate. 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. In the solution competition approach (also called inhibition assay), a known amount of a 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.

Calibration-free assays

- 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-controlled conditions. This approach can be useful in situations where no satisfactory calibrant is available for the analyte under study. Calibration-free assays are always set up in the direct binding format.

The main formats are illustrated schematically in Figure 3-1, and described in more detail in the sections that follow.
3 Assay formats
3.1 Calibrated direct binding assays

Figure 3-1. Schematic illustration of four different approaches to concentration measurement with Biacore.

3.1 Calibrated direct binding assays

Direct binding assays represent the most direct approach to measuring concentration with Biacore. The detecting molecule is attached to the sensor chip surface: sample is injected, and either the binding rate or the response obtained after a fixed contact time is measured (Figure 3-2). The response is related to analyte concentration with the help of a standard curve, prepared by analyzing known concentrations of analyte under the same conditions.
3.1.1 Single step response level measurement

Direct measurement is suitable for macromolecular analytes (molecular weight >5000 daltons) which give an easily measured response even at low molar concentrations.

The response in Biacore is a measure of the refractive index change at the surface of the sensor chip, and does not distinguish between analyte binding to the ligand and differences in the bulk refractive index between sample and running buffer. For this reason, binding levels for concentration measurement are most reliably measured shortly after the end of the sample injection, when running buffer is flowing over the surface and the response increase from the baseline before the injection reflects only bound analyte (see Section 7.2.2).
The duration of the sample injection should ideally be long enough to allow the sensorgrams to flatten out to some extent, although it is not necessary for the binding to reach a steady state. If the injections are too short, the sensitivity of the assay may be impaired (Figure 3-4): in addition, the robustness of the assay will be critically dependent on the exact placing of the report point if binding is rapid.

![Figure 3-4. A single step direct assay gives an increasing response with increasing concentration. An interaction that is allowed to approach equilibrium will give a higher sensitivity at low analyte concentrations.](image)

### 3.1.2 Binding rate measurements

The rate of analyte binding during sample injection is directly related to the analyte concentration, and can be used instead of the response level as the basis for a direct binding assay. Binding rates should generally be measured shortly after the start of the sample injection, since the rates approach zero as the binding approaches a steady state. This approach may be useful if the analyte dissociates rapidly from the surface after the end of the injection, since the binding rate (measured as the slope of the sensorgram) is not affected by the contribution of bulk refractive index to the response (see Figure 3-2).

### 3.1.3 Sandwich methods

Sandwich methods are an extension of the single step direct approach: after analyte has bound to the surface-attached ligand, a secondary interactant (the enhancement molecule) is injected to bind to the analyte and enhance the analyte response. If the secondary analyte is a larger molecule than the analyte or binds to multiple sites (such as a polyclonal antibody preparation), the secondary response will be proportionally higher than the primary response.
obtained from the analyte itself, providing an amplification of the signal (Figure 3-5).

**Figure 3-5.** Sensorgrams (inset) and calibration curves for a direct binding assay with enhancement. ①: primary response (analyte), ②: secondary response (enhancement reagent).

In addition to the signal amplification which can improve the limits of detection and quantitation, sandwich methods can provide enhanced specificity, since the measured secondary response reflects the combined specificities of analyte binding to detecting molecule and to secondary interactant. This can be valuable in measuring concentrations in the presence of non-specific binding, where the secondary response confirms the identity of the molecule detected in the first interaction. It also finds application in measuring the concentration of bifunctional molecules, in particular recombinant tagged proteins where one interaction may be directed against the tag and the second interaction identifies the target molecule.

A prerequisite for sandwich methods is that the analyte has distinct, non-interfering binding sites for the detecting molecule and the secondary interactant. In practice, this restricts the usefulness of the approach to macromolecular analytes.

Sandwich assays are part of the standard repertoire of immunological assay techniques in other formats such as RIA and ELISA. However, these techniques only measure the secondary interactant (the radio- or enzyme-labeled antibody that detects analyte bound to the immunoassay substrate). A significant difference in the Biacore-based approach is that binding data are obtained automatically for both the primary and secondary interactions. It is therefore a simple matter to construct standard curves based on both primary and secondary responses.
3.2 Calibrated indirect assays

The response obtained in Biacore is a measure of the change in mass concentration of solutes close to the sensor surface. Accordingly, low molecular weight (LMW) analytes give a lower response than macromolecules for an equivalent molar concentration. While binding of molecules as small as 200 daltons or less can be observed under favorable circumstances, the usefulness of direct binding level assays for measuring the concentration of small molecules is limited. Indirect assays represent an alternative approach in such cases.

Competition assays work on the principle of allowing a high molecular weight (HMW) detecting molecule to bind to the sensor surface in competition with the analyte. The response, which measures the amount of HMW molecule bound, gives an inverse measure of the concentration of analyte in the sample. Two different formats are generally recognized, according to whether the detecting molecule is in solution or on the surface.

3.2.1 Inhibition assays

Inhibition assays, also called solution competition, exploit the ability of the analyte to inhibit the binding of HMW detecting molecule to the surface (Figure 3-1). Typically, the analyte or a derivative thereof is attached to the surface as the ligand, while the detecting molecule is a macromolecule that binds specifically to the analyte. A constant amount of detecting molecule is added to the samples. The mixture is incubated to reach equilibrium, and then injected over the sensor surface to measure the remaining free detecting molecule. The amount of free detecting molecule is inversely related to the concentration of analyte in the sample (Figure 3-6). Inhibition assays are most commonly used for low molecular weight analytes.

Clearly, it is necessary that detecting molecules carrying bound analyte in solution should not be able to bind to the surface-attached ligand. Ideally, the HMW detecting molecule should be monovalent for this reason. However,
monoclonal antibodies are commonly used as detecting molecules, in spite of their bivalent binding properties. Even though both antigen-binding sites must be occupied to effectively inhibit the binding of antibody to the surface, the inhibition assay principle still works reliably and antibodies are often the most readily available source of detecting molecules.

The affinity of the detecting molecule for the analyte in solution together with the concentration of detecting molecule determines the useful range of an inhibition assay (Figure 3-7). Higher affinities allow measurement at lower analyte concentrations but also result in a narrower operating range (Figure 3-7). In formal terms, the IC\textsubscript{50} of an inhibition assay (the concentration that gives 50% inhibition) is given by

\[ \text{IC}_{50} = K_D + \frac{C}{2} \]

where \( K_D \) is the equilibrium dissociation constant for the interaction with analyte and \( C \) is the total concentration of detecting molecule. If the concentration is much smaller than the dissociation constant, the range will be determined primarily by the affinity; conversely, if the concentration is much higher than the dissociation constant, the range will be determined primarily by the concentration.

**Figure 3-7.** Increasing the affinity of the detecting molecule moves the operating range to lower analyte concentration and also narrows the range.

### 3.2.2 Surface competition

In the surface competition approach, a binding partner to the analyte is used as ligand, and a high molecular weight analogue to the analyte (typically analyte conjugated to a carrier protein) is added in constant amount to the samples to be measured. The basis of the assay is competition between analyte and the
3 Assay formats
3.3 Calibration-free concentration assays

HMW analogue for binding to the ligand (Figure 3-1). The measured response is the sum of the contributions from analyte and HMW molecule: as with the solution competition approach, the response is inversely related to the amount of analyte in the sample (see Figure 3-6). In the same way as for direct binding assays, it is not necessary for the interaction of analyte or analogue with the surface to reach a steady state for surface competition assays. This contrasts with inhibition assays, where the interaction of analyte with the detecting molecule in solution should reach equilibrium before the sample is injected over the surface.

The surface competition approach can have advantages over the more common inhibition assay format in situations where immobilization of the analyte on the sensor chip surface presents problems. LMW analytes that are typically addressed with competition assays are not always amenable to the chemistry developed for attaching macromolecular ligands to the surface, and development of a suitable immobilization method for analyte which preserves the ability to bind detecting molecule can be a time-consuming process. It can in many cases be simpler to develop a method for conjugating the analyte molecule to a carrier protein. The properties of the carrier protein are not important, provided that (a) it is large enough to give a response clearly distinguishable from that given by analyte alone, (b) it does not bind on its own to the sensor chip surface with attached ligand and (c) the conjugated analyte molecules are still available for binding to the ligand. The level of conjugation should be kept low (an average of one or less analyte molecules per carrier protein) to avoid avidity effects that arise from multiple ligand binding sites. This gives considerable freedom in the choice of carrier molecule: frequently a cheap and readily available protein like transferrin or haptoglobin is fully adequate for the purpose. Avoid using serum albumin for this purpose since it binds to many small molecules.

3.3 Calibration-free concentration assays

Calibration-free concentration measurements rely on measurement of the rate of diffusion (also called mass transport) of analyte from bulk solution to the sensor surface, and calculation of the absolute analyte concentration from this rate and a knowledge of the diffusion properties of the analyte molecule. The mass transport rate is determined by fitting the observed binding data to a kinetic interaction model that includes a term for mass transport. Details of the data analysis are given in Appendix B.

3.3.1 Scope and limitations of calibration-free assays

Calibration-free assays can be particularly useful in situations where no satisfactory calibration standard is available for the analyte, or as a check on the validity of the specified concentration in standards.
The approach is however subject to some limitations:

- The method can only be used for proteins with molecular weight above 5000 Da.
- Analyte-ligand interactions with slow association (association rate constant $k_a$ less than about $5 \times 10^4$ M$^{-1}$s$^{-1}$) and/or low affinity (equilibrium dissociation constant $K_D$ higher than about $10^{-6}$ M) are generally not suitable.
- The method cannot handle measurements on mixtures of analytes with different diffusion properties. For example, the approach can be used to determine the total concentration of a mixture of IgG antibodies in a polyclonal preparation, since all IgG molecules are essentially the same size and shape, but it cannot be used to determine the total antibody concentration in a mixture containing IgG together with other antibodies of different size such as IgM or IgE.
- The dynamic range of the method is approximately 0.05–5 µg/ml.

### 3.3.2 Setting up calibration-free assays

 Calibration-free concentration measurements are directly supported in some Biacore systems, but may in principle be applied in all systems that permit measurement of interaction kinetics under partially mass transport-limited conditions.

 The recommended approach involves analysis of the interaction with the same sample during short sample injections (recommended 36 s) using at least two different flow rates. In practice, flow rates as widely separated as the system will allow are normally recommended (e.g. 5 and 100 µl/min). A blank cycle (injecting buffer instead of sample) is recommended for each flow rate: subtraction of this blank from the sample cycles helps to eliminate systematic variations in the response and improves the robustness of the assay.

 Calibration-free measurements should always be performed on Sensor Chip CM5. This is partly to facilitate immobilization of sufficiently high ligand levels, but also because the evaluation depends on a conversion between RU and surface concentration (see Appendix B), which may not be valid for other sensor chips.

 Levels of immobilized ligand should be high (typically 5,000–10,000 RU for proteins) to favor mass transport-limited interaction.

 For best results, the initial binding rate at 5 µl/min should be between about 0.3 and 15 RU/s (corresponding to concentrations for typical proteins in the approximate range 0.05–5 µg/ml), and the ratio of the binding rates at 100 and 5 µl/min should be higher than about 1.3. The sensorgram at the lower flow rate...
should be approximately linear during the first 30 seconds of interaction. Evaluation will be less robust if these conditions are not met.

Considerations for choice of ligand for calibration-free assays are essentially the same as for direct binding assays using a calibration curve (Section 3.5.2).

Details of how calibration-free concentration assays are evaluated are given in Appendix B.

### 3.4 Choice of assay technique

As discussed above, Biacore offers a number of different approaches to measuring concentration. The choice of approach is dictated largely by the characteristics of the analyte being assayed, and to some extent by the purpose of the assay and the type of sample matrix that will be used. The table below provides some general guidelines.

| Macromolecules (typically MW >5000 daltons) | Direct assay with the detecting molecule attached to the sensor surface. Sandwich assay can help to
sandwich assay can help to
- amplify the response for molecules at the low end of the size range
- confirm the identity of the analyte in complex mixtures
- confirm the integrity of multifunctional analytes.
| Small molecules (typically MW <5000 daltons) | Inhibition assay with the analyte or analogue attached to the surface and detecting molecule in solution.
| Small molecules which are difficult to attach to the sensor surface | Competition assay with the detecting molecule attached to the sensor surface and an analyte conjugate in solution.
| Macromolecular analytes for which no acceptable calibration standard is available | Calibration-free assay, provided that a value for the diffusion coefficient is available (diffusion coefficients can also be estimated from the molecular weight and shape properties of the molecule). |

For all approaches, the choice of detecting molecule is dictated primarily by the availability of a suitable molecule, although the purpose of the assay can be important in some cases. Antibodies are a common choice because they are readily available, specific for the analyte, and offer a range of affinities for adjusting the operating range of the assay (see Section 7.3). Sometimes however it may be more appropriate to choose a detecting molecule related to the functional activity of the analyte. An assay for measuring antibody
concentration provides an illustrative example: if the ligand binds to the Fc portion of an IgG molecule, the assay will measure total IgG concentration. However, if the antigen is used as ligand, the assay will measure only the concentration of antigen-specific antibody.

3.5 Choice of ligand and other reagents

Depending on the design of the assay, there may be a range of possible ligands. Immunological assays typically offer a panel of antibodies that may be used as ligands or detecting molecules. On the other hand, assays based for example on the binding of analyte to a specific cell surface receptor are limited in their choice of detecting molecule.

In general, ligand and other reagents are selected on the basis of known interaction properties, but the suitability of the selection always needs to be confirmed in practice. If details of the interaction properties are not known in advance, selection is done entirely through practical tests.

3.5.1 Practical approaches to reagent selection

To confirm the suitability of a selected reagent, perform measurements using samples with known analyte concentrations that cover at least the intended range of the assay. Construct a calibration curve for the assay as a tool for deciding whether the reagent choice is acceptable.

Measuring a calibration curve with known analyte samples presupposes that assay conditions have been established, including conditions for surface preparation (Chapters 4 and 5) and regeneration (Chapter 6). The process of selecting and optimizing reagents is necessarily iterative, since all steps in the assay set-up and execution need to be confirmed before the assay as a whole can be approved. A reagent that is acceptable in terms of interaction properties may for instance be discarded on the grounds of difficulties in regeneration. Be prepared to review your choice of ligand or other reagents on the basis of results from later steps in the assay development procedure.

3.5.2 Direct binding assays

Choice of ligand

For direct binding assays, base the choice of ligand on the following considerations:

- The ligand should if possible be available in a purified state with a high specific activity (i.e. binding capacity for the analyte). If partially purified ligand preparations are used, higher total amounts of material must be immobilized to achieve the same surface binding capacity, and additional control experiments will be necessary to establish the assay performance.
3 Assay formats
3.5 Choice of ligand and other reagents

- The ligand should be amenable to immobilization at high levels without serious loss of analyte binding capacity.
- The interaction of ligand with analyte should show selectivity characteristics that are compatible with the demands of the assay. Any lack of specificity in the ligand-analyte binding will be reflected in a low selectivity for the assay.
- The binding of analyte to ligand should be fairly rapid so that analysis cycle times can be kept short, and should be relatively stable so that response levels can be measured after sample injection to avoid complications from bulk refractive index effects. In practice this means that the ligand should have a high affinity for the analyte. The affinity of ligand for analyte may be a determining factor in the operating range of the assay: a higher affinity interaction can often be used to measure lower analyte concentrations.
- For calibrated assays based on binding rates, the association rate must be within a reliably measurable range (rate constant \( k_a \) typically in the range \( 5 \times 10^3 \) – \( 5 \times 10^6 \) M\(^{-1}\)s\(^{-1}\), although the usable range will depend on the required concentration range of the particular assay). For calibration-free assays, \( k_a \) should be higher than \( 5 \times 10^4 \) M\(^{-1}\)s\(^{-1}\) to ensure conditions for adequate mass transport limitation.
- It must be possible to regenerate the ligand efficiently without loss of ligand activity (see Chapter 6). If a compromise is necessary between regeneration characteristics and affinity for the analyte, regeneration characteristics are more important than high affinity.

Enhancement reagent

The enhancement reagent used in sandwich assay formats must be able to bind to the analyte at a site independent of the ligand binding site. Antibodies are most commonly used as enhancement reagents, but in principle any macromolecule that can bind to analyte independently of ligand can be used. Knowledge of the binding site topology on the analyte (termed epitope mapping in immunological contexts) can help to restrict the available choice of enhancement reagent.

Enhancement reagents intended primarily to amplify the analyte response should either be larger molecules than the analyte or bind to multiple independent sites on the analyte. Reagents used to enhance specificity, on the other hand, do not necessarily have to be larger than the analyte provided that a confidently measurable signal is obtained.

High affinity for the analyte is an advantage for enhancement reagents, since maximum response can be obtained with short contact times.
3.5.3  Inhibition assays

The ligand for inhibition (solution competition) assays is the same as the analyte or an analogue thereof. The choice of ligand is thus given, with a certain degree of freedom in finding or synthesizing analogues that are suitable for immobilization on the surface.

Demands on the detecting molecule for inhibition assays are broadly similar to the demands on ligand for direct binding assays. The interaction between detecting molecule and analyte should be moderately fast, so that incubation times of sample with detecting molecule and contact times of injection over the surface are kept reasonably short in the interests of assay throughput.

3.5.4  Surface competition assays

The ligand in surface competition assays is analogous to that in direct binding assays and the choice of molecule is subject to the same considerations. In addition, it is necessary that the analyte and competing molecule each bind to the ligand to the exclusion of the other, so that a true competition situation is achieved. The relative affinities of analyte and competing molecule will determine to some extent the range of analyte concentrations that can be measured.

The competing molecule for surface competition assays should preferably be much larger than the analyte, so that the observed response derives almost exclusively from binding of the competing molecule. If binding of the analyte itself gives rise to a significant response, assay performance may be impaired. (In the extreme case where analyte and competing molecule are the same size, there will be no change in observed response as analyte displaces the competing molecule from the surface.)
3 Assay formats
3.5 Choice of ligand and other reagents
4 Surface preparation principles

Measurements with Biacore are based on interaction of analyte in solution with ligand attached to the sensor chip. Molecules can be attached to the chip surface either covalently using a variety of chemical methods or through high affinity capture to a specific capturing molecule (which is in turn attached chemically to the surface).

This chapter gives an overview of the methods available for attaching the ligand to the sensor chip surface. Practical procedures are described in the next chapter.

A range of sensor chips is available for use in Biacore. The most versatile is Sensor Chip CM5, a general-purpose chip with a high surface capacity that supports a wide range of ligand immobilization chemistries. Information in this chapter relates to Sensor Chip CM5 unless otherwise stated. Details of other surfaces may be found in the Biacore Sensor Surface Handbook or obtained from GE Healthcare.

4.1 Sensor surface properties

The sensor chip is a glass slide coated with a thin layer of gold, to which a matrix of carboxymethylated dextran is covalently attached (Figure 4-1). The gold is required for generation of the SPR response (Appendix A). Ligands can be attached to the dextran matrix using a variety of chemical methods.

![Figure 4-1. Schematic illustration of the structure of the sensor chip surface.](image)

The gold layer and dextran matrix on the sensor surface are stable under a wide range of conditions, including extremes of pH and moderate concentrations of many organic solvents. Once the ligand has been immobilized, the stability of the sensor surface is determined primarily by the stability of the attached ligand.
4 Surface preparation principles
4.2 Ligand immobilization methods

4.2 Ligand immobilization methods

The carboxymethylated dextran matrix on the sensor surface is amenable to a range of chemistries for ligand immobilization, exploiting different groups on the ligand molecule. Immobilization approaches may be directed towards amine, carboxyl, thiol or hydroxyl groups on the ligand, or may use specific tags introduced into the ligand either by chemical modification or recombinant techniques.

Two basic chemical approaches are recommended for general use:

- Amine coupling, using primary amine groups on the ligand.
- Thiol coupling, using exchange between reactive disulfides and native or introduced thiol groups on the ligand.

Additional methods that can be valuable in situations where amine or thiol coupling is unsatisfactory include:

- Aldehyde coupling, using aldehyde groups on the ligand (introduced through oxidation of cis-diols, typically in carbohydrate residues).
- Irreversible high affinity capture of biotinylated ligand on immobilized streptavidin.
- Reversible high affinity capture by binding to specific capturing molecules (e.g., high affinity antibodies). Note that it is often difficult to achieve the high ligand levels recommended for concentration measurements with a reversible capturing approach. In addition, fresh ligand is captured for each analysis cycle, so that ligand consumption is high and minor variations in amount captured between cycles may impair assay performance.

The chemistry involved in the different approaches is summarized below.

4.2.1 Amine coupling

Amine coupling chemistry is the most widely applicable approach for attaching biomolecules covalently to the sensor surface. With this method, the dextran matrix on the sensor chip surface is first activated with a mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to give reactive succinimide esters. Ligand is then passed over the surface and the esters react spontaneously with uncharged amino groups or other nucleophilic groups to link the ligand covalently to the dextran (see Figure 4-2).
Most proteins contain several amine groups so that efficient attachment can be achieved without seriously affecting the biological activity of the ligand. In some instances, however, amine coupling may involve groups at or near the active site or binding site of the ligand, with the result that attachment is accompanied by loss of activity. In such cases, the ligand can be attached using alternative coupling chemistry or a capturing approach.

### 4.2.2 Thiol coupling

Thiol coupling utilizes exchange reactions between thiol and active disulfide groups. The active disulfide moiety may be introduced either on the dextran matrix (to exchange with a thiol group on the ligand, referred to as the ligand thiol approach) or on the ligand molecule (to exchange with a thiol group introduced on the dextran matrix, referred to as the surface thiol approach). A recommended reagent for introducing active disulfide groups is 2-[2-pyridinylthio]ethaneamine (PDEA, Figure 4-3). The amine group in PDEA can be used to attach the molecule to activated carboxyl groups on either the surface or the ligand. Figure 4-4 illustrates the two thiol coupling approaches.

![Figure 4-3. PDEA thiol coupling reagent, 2-[2-pyridinylthio]ethaneamine hydrochloride.](image)
4 Surface preparation principles
4.2 Ligand immobilization methods

Thiol coupling can be a valuable approach if the ligand is inactivated by amine coupling as a result of the presence of an active amine group in the analyte binding site. The thiol approach can also help to immobilize ligands in a defined orientation, since the number of potential attachment sites is often less than with amine coupling, and in many cases is reduced to one single site. Surface thiol coupling is also valuable for acidic proteins, since the substitution with PDEA raises the isoelectric point of the protein, improving the electrostatic pre-concentration properties (see Section 4.3.1).

4.2.3 Aldehyde coupling

Ligands containing aldehyde groups (either native or introduced by oxidation of cis-diols) can be immobilized after activating the surface with hydrazine or carbohydrazide. The chemistry of aldehyde coupling is shown in Figure 4-5.

Aldehyde coupling provides an alternative approach for immobilizing glycoproteins and other glycoconjugates. The method is particularly suitable for ligands containing sialic acid, since these residues are very easily oxidized to aldehydes.
4.2.4 Streptavidin-biotin capture

Streptavidin immobilized on the sensor chip surface can be used to capture biotinylated ligands with high efficiency. The affinity of streptavidin for biotin is very high (K_D of the order of 10^{-15} M), so that the ligand is in practice permanently attached to the surface. Sensor Chip SA is a sensor chip with streptavidin pre-immobilized on the surface for capture of biotinylated ligands.

**Notes:** Ligand immobilization through streptavidin-biotin interaction is technically a capturing approach. However, the interaction affinity is so high that the captured ligand cannot be removed without destroying the surface. In practice, therefore, this method resembles covalent immobilization rather than capturing.

In some cases, the affinity of the biotinylated ligand for streptavidin may be significantly lower than that of free biotin. Do not use streptavidin-biotin capture if the sensorgram baseline shows significant downward drift, indicating loss of ligand from the surface.

A range of methods and reagents for ligand biotinylation is available: the choice of method will depend largely on the nature of the ligand. Reagents with long spacer arms can be used to separate the biotin residue from the ligand molecule and reduce steric hindrance effects in immobilization and analysis. Substitution levels of 1-2 biotin residues per ligand molecule are recommended for capture on streptavidin. In general, the conditions recommended with commercial biotinylation reagents tend to give higher substitution levels, resulting in multi-point attachment of the ligand to the surface with consequent impairment of assay performance.

4.2.5 General capture methods

Immobilization of ligand by capturing involves high affinity binding of the ligand to an immobilized capturing molecule (Figure 2-1). In general, the regeneration step in the assay procedure removes ligand along with any remaining bound analyte, so that new ligand needs to be captured for each assay cycle.

Capturing approaches can provide an alternative to covalent immobilization in situations where it is difficult to find chemical methods that give satisfactory results, or where the assay requires that the ligand on the surface can be changed. Capturing can also provide a ligand purification step, for example when tagged recombinant ligands are captured from partially purified material through binding of the tag to a specific capturing molecule.

The basic requirement for successful capturing is a robust high affinity interaction between the capturing molecule and the ligand. Monoclonal antibodies are frequently used as capturing agents. Even if the affinity is high, there will often be a certain amount of dissociation of the ligand from the
4 Surface preparation principles
4.3 Conditions for ligand immobilization

surface during the course of an analysis. Since ligand is refreshed for each cycle, this can generally be ignored in concentration assays.

The capturing molecule is attached to the surface using one of the covalent chemical approaches described in the previous sections.

4.2.6 Immobilizing small molecules

Some assay formats, in particular inhibition assays, involve immobilization of small molecules on the sensor surface. The general principles for immobilizing small molecules are the same as for macromolecules, but there are a number of differences in practice:

- Small molecules do not in general offer the same diversity of potential attachment sites as macromolecules. In some cases, a suitable amine or thiol group may be present in the native molecule: in others it may be necessary to introduce such a group through modification of the molecule.

- The chemical procedure of immobilizing a small molecule involves a greater risk of adversely affecting its binding to the interaction partner, since the chemical modification affects a larger proportion of the molecular structure. In many cases, it can be advantageous to introduce the coupling group on a spacer arm so that the immobilization reaction is kept at a distance from the functional molecule.

- Electrostatic pre-concentration (see Section 4.3.1) is usually ineffective for small molecules. High concentrations (typically 5-10 mM) are used in immobilization to compensate for this effect.

- Capturing approaches cannot normally be used for small molecules, since they require that the molecule in question carries independent binding sites for the capturing molecule and the analyte. It may however in some cases be feasible to construct a bifunctional entity where one moiety binds to the capturing molecule and the other interacts with the analyte, for instance by linking the ligand to a biotin moiety.

4.3 Conditions for ligand immobilization

Considerations in this section apply to covalent immobilization of macromolecules on the sensor surface. For ligand capture, the capturing molecule is first covalently immobilized according to the considerations described here. Subsequent capture of the ligand is an interaction process between the capturing molecule and the ligand.
4.3.1 Buffer conditions and pre-concentration

At the concentrations of ligand used for immobilization (typically of the order of 10-50 µg/ml), levels of immobilized ligand would be low in the absence of a mechanism for concentrating the ligand molecules at the surface. Electrostatic attraction of the ligand to the surface is the main mechanism for this concentration process. This attraction is referred to as pre-concentration, and can result in a several thousand-fold concentration of ligand on the surface.

Buffer pH

The carboxymethylated surface of the sensor chip carries a net negative charge at pH values above about 3.5, so that to achieve efficient pre-concentration the pH of the buffer should be higher than 3.5 and lower than the isoelectric point of the ligand. The optimum pH may be a compromise between efficient pre-concentration and the stability of the ligand, and choice of pH can in some cases be a critical parameter in determining the success of immobilization.

Acidic proteins with isoelectric points below about 4.5 can be difficult to immobilize efficiently, because of the narrow pH interval in which the protein is positively charged and the surface is negatively charged. Modification of carboxyl groups on the ligand with PDEA as the first step in surface thiol coupling chemistry (Section 4.2.2) can be an advantage in this respect, since blocking of the carboxyl groups on the ligand molecule with PDEA usually raises the isoelectric point. In situations where effective pre-concentration cannot be achieved, high affinity capturing methods may provide an alternative approach.

Ionic strength

An additional requirement for efficient pre-concentration is a low ionic strength in the immobilization buffer. In general, a maximum of 10-15 mM monovalent ions is recommended. Some proteins show limited stability under such conditions, so that ligand solutions for immobilization should be prepared...
immediately before use. Increase the ionic strength in the immobilization buffer only if this is absolutely necessary.

Sodium acetate (10 mM) is generally recommended as a buffer for immobilization over the pH range 4.0–5.5.

**Ligand concentration**

The concentration of ligand required for immobilization is generally low provided that pre-concentration is efficient. For most proteins, concentrations of 10–50 µg/ml are sufficient, and higher concentrations simply consume more ligand without significantly improving the results. Lower concentrations may be used in favorable cases.

Higher ligand concentrations may be used to improve pre-concentration if necessary. However, poor pre-concentration can never be fully compensated by increased ligand concentration, since the final volume concentration in the surface layer is many times higher than that in solution (a response level of 1000 RU corresponds to a protein concentration of roughly 10 mg/ml in the surface layer).

### 4.4 Strategy for surface preparation

#### 4.4.1 Amount of immobilized ligand

The general guideline for concentration assays is to immobilize as much ligand as is reasonably possible within the constraints of available ligand concentration, contact times etc (see Chapter 5 for practical details). For protein ligands of average molecular size (of the order of 50,000-150,000 daltons), immobilization levels of 7,000-15,000 RU are typical. The analyte binding response at a given sample concentration is directly related to the level of immobilized ligand, so that a high immobilization level will enable measurements at lower analyte concentrations. In addition, high levels of immobilized ligand ensure rapid binding of analyte and favor mass-transport limited binding (see Appendix B), making concentration measurements less dependent on the affinity of ligand for analyte.

The level of immobilized ligand may need to be kept lower in assays that use capturing or enhancement formats, where a multi-molecular complex is built up on the sensor surface during the course of an assay. If several of the components are large, high levels of immobilized ligand can result in crowding and steric hindrance between binding molecules at a subsequent stage in the assay, limiting the observed response.
4.4.2 Choice of immobilization method

For most protein ligands, amine coupling is the simplest approach, although it is not necessarily the most effective. For some proteins, thiol coupling can give better yields of immobilized ligand. Acidic proteins which are not efficiently pre-concentrated in their native state can be modified with PDEA for surface thiol coupling: this reaction modifies carboxyl groups on the protein, raising the isoelectric point of the protein and facilitating immobilization.

In cases where amine or thiol coupling is not satisfactory, either because yields are too low or because the coupling procedure inactivates the protein, biotinylation followed by capture on a streptavidin surface can often provide an alternative approach. Biotinylation can be performed using mild reaction conditions.

Capture through streptavidin-biotin interaction is also the method of choice for immobilizing nucleic acids, which are easily biotinylated and which are generally not amenable to amine or thiol coupling chemistry. In addition, capturing methods are much less dependent on electrostatic pre-concentration, which is inefficient for nucleic acids.

Some biomolecules, notably carbohydrates and glycoconjugates, may be successfully immobilized using aldehyde chemistry after oxidation of cis-diols in the ligand to aldehydes.
4  Surface preparation principles
4.4  Strategy for surface preparation
5 Surface preparation in practice

Surface preparation involves essentially three separate steps, described in more detail in the sections that follow. Some Biacore systems offer software wizards that provide assistance in setting up the practical procedures.

- Immobilization pH scouting, to determine a suitable pH and concentration for ligand immobilization.
- Attachment of the ligand to the surface.
- Testing the activity of the surface to establish that analyte binds satisfactorily to the immobilized ligand.

The guidelines in this chapter apply to immobilization of ligand directly on the sensor chip surface. When ligand is captured through a high affinity interaction (see Section 4.2.5), the capturing molecule is immobilized as described here, and ligand is then injected in buffer over the surface, usually as the first step in each analysis cycle.

5.1 Conditions for immobilization

Temperature
Surface activation and ligand attachment should normally be performed at 25°C unless the ligand is temperature-sensitive. Immobilization at lower temperatures may require prolonged contact times for surface activation and ligand immobilization. Using higher temperatures may in some cases help to increase the immobilization level obtained.

Ligand concentration
Ligand solutions for immobilization are quite dilute (typically 10-50 µg/ml for most proteins). Higher concentrations may be needed for proteins that for reasons of low isoelectric point or other factors do not attach efficiently to the surface.

Buffer conditions
For many proteins, coupling in 10 mM acetate buffer pH 4.5 works well. If you need to use other conditions, bear the following considerations in mind:

- The buffer pH should be at least 0.5–1 unit below the isoelectric point of the ligand. For ligands with isoelectric point above pH 7, the buffer pH may be increased to 5.5 or 6.
- The ionic strength should be low (recommended 10–20 mM monovalent cations).
5 Surface preparation in practice
5.1 Conditions for immobilization

- Buffer components containing primary amine groups and other strong nucleophilic groups (e.g. Tris, sodium azide) must be avoided for amine coupling, since these will compete with the ligand for activated esters on the sensor chip surface. Thiol coupling must be performed in the absence of reducing agents.

Many proteins are relatively unstable at low pH and low ionic strength, so ligand solutions for coupling should be prepared shortly before use. If the ligand is stored in a stock solution at a different pH and/or higher ionic strength, it is important that the transfer of ligand to coupling buffer is performed correctly. If the stock concentration is not high enough to allow the stock buffer to be “diluted out”, a buffer exchange technique is recommended (e.g. desalting column, dialysis etc).

**Determining suitable coupling conditions**

In order to determine suitable coupling conditions without permanently modifying the sensor chip surface, inject ligand in coupling buffer over a surface that has not been activated with EDC/NHS, using a contact time of 2 minutes. Non-covalent electrostatic binding of ligand to the surface (pre-concentration) will be seen as an increase in response, and will give an indication of whether the conditions are suitable. As a general rule, the ligand response should reach at least 5000 RU above the baseline within 2 minutes of sample injection.

The procedure below outlines the steps in determining a suitable coupling pH. An analogous procedure may be used to test other aspects of the coupling conditions.

1. Prepare ligand solutions in the different coupling buffers to be tested. For many proteins, pH 4.5 works well, but you may need to scout for a different pH value if pre-concentration at pH 4.5 is not satisfactory.
2. Inject the ligand solution, using a contact time of 2 minutes.
3. Set report points just before the start and end of the injection to determine the level of electrostatically bound ligand.
4. Inject a short pulse of 1 M ethanolamine-HCl pH 8.5 (included in the Amine Coupling Kit) or 50 mM NaOH over the surface to remove the last traces of electrostatically bound ligand.
5. If necessary, repeat steps 2 and 3 with different ligand solutions.

In assessing the results of pre-concentration tests, bear in mind that electrostatic binding is generally more efficient at lower pH values, but the amine coupling chemistry requires uncharged amine groups and is therefore more efficient at higher pH. Choose the highest pH that gives adequate pre-concentration.
If you do not obtain satisfactory electrostatic pre-concentration at any pH, try increasing the ligand concentration. If this does not help, you may need to use a different coupling approach.

5.2 Immobilization procedure

The general pattern of steps involved in immobilizing ligand is as follows:

1. Activate the surface by injection of appropriate reagents.
2. Inject the ligand solution.
3. Inject reagent to deactivate remaining active groups on the surface and remove non-covalently bound ligand.

There may be additional steps depending on the properties of the ligand, the type of sensor surface and the details of the chemistry used. Different Biacore systems provide varying levels of software wizard support for ligand immobilization.

5.2.1 Preparing solutions

For most immobilization approaches, the surface is activated with a mixture of 0.05 M EDC and 0.2 M NHS (final concentrations). Reagent solutions should be freshly prepared or stored frozen and mixed shortly before use. The efficiency of immobilization will be reduced if the solutions are not fresh.

Ligand solutions should be prepared in immobilization buffer shortly before use, following the guidelines in Section 4.3.

5.2.2 Amine coupling

The chemistry of amine coupling is described in Section 4.2.1.

Required solutions

Reagents may be obtained from GE Healthcare as Amine Coupling Kit.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDC</td>
<td>0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in water</td>
</tr>
<tr>
<td>NHS</td>
<td>0.1 M N-hydroxysuccinimide in water</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>1 M ethanolamine-HCl pH 8.5</td>
</tr>
<tr>
<td>Ligand</td>
<td>Typically 10-50 µg/ml in immobilization buffer</td>
</tr>
</tbody>
</table>
5.2 Immobilization procedure

Recommended immobilization protocol

<table>
<thead>
<tr>
<th>Injection</th>
<th>Flow rate</th>
<th>Contact time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. EDC/NHS (activate the surface)</td>
<td>10 µl/min</td>
<td>7 min</td>
</tr>
<tr>
<td>2. Ligand</td>
<td>10 µl/min</td>
<td>7 min</td>
</tr>
<tr>
<td>3. Ethanolamine (deactivate excess reactive groups)</td>
<td>10 µl/min</td>
<td>7 min</td>
</tr>
</tbody>
</table>

5.2.3 Surface thiol coupling

The chemistry of surface thiol coupling is described in Section 4.2.2. The ligand is modified first to introduce reactive disulfide groups. A recommended procedure for modification of carboxyl groups on the ligand with PDEA is described below.

**Modification of the ligand with PDEA**

Prepare a solution of 1 mg protein and 5.5 mg PDEA (final concentration 25 mM) in 1 ml 0.1 M MES buffer pH 5.0. Cool on ice, then add 50 µl 0.4 M EDC in water. Incubate on ice for 1 hour. Remove excess reagents by gel filtration or similar technique.

**Immobilization - required solutions**

EDC and NHS are included in Amine Coupling Kit from GE Healthcare. PDEA is available from GE Healthcare.

<table>
<thead>
<tr>
<th>EDC</th>
<th>0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in water</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHS</td>
<td>0.1 M N-hydroxysuccinimide in water</td>
</tr>
<tr>
<td>Cystamine</td>
<td>0.04 M cystamine dihydrochloride in 0.1 M sodium borate pH 8.5</td>
</tr>
<tr>
<td>DTE</td>
<td>0.1 M dithioerythritol in 0.1 M sodium borate pH 8.5. Dithiothreitol (DTT) may also be used</td>
</tr>
<tr>
<td>PDEA/NaCl</td>
<td>20 mM 2-(2-pyridinyl)dithiolethaneamine and 1 M NaCl in 0.1 M sodium acetate pH 4.3</td>
</tr>
</tbody>
</table>
5.2.4 Ligand thiol coupling

The chemistry of ligand thiol coupling is described in Section 4.2.2.

Recommended immobilization protocol

<table>
<thead>
<tr>
<th>Injection</th>
<th>Flow rate</th>
<th>Contact time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. EDC/NHS (activate the surface)</td>
<td>10 µl/min</td>
<td>2 min</td>
</tr>
<tr>
<td>2. Cystamine (introduce disulfide groups)</td>
<td>10 µl/min</td>
<td>3 min</td>
</tr>
<tr>
<td>3. DTE (reduced disulfides to thiols)</td>
<td>10 µl/min</td>
<td>3 min</td>
</tr>
<tr>
<td>4. Ligand</td>
<td>10 µl/min</td>
<td>7 min</td>
</tr>
<tr>
<td>5. PDEA-NaCl (deactivate excess reactive groups)</td>
<td>10 µl/min</td>
<td>4 min</td>
</tr>
</tbody>
</table>

Required solutions

EDC and NHS are included in Amine Coupling Kit from GE Healthcare. PDEA is available from GE Healthcare.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDC</td>
<td>0.4 M 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide in water</td>
</tr>
<tr>
<td>NHS</td>
<td>0.1 M N-hydroxysuccinimide in water</td>
</tr>
<tr>
<td>PDEA</td>
<td>80 mM 2-(2-pyridinyl)dithiolethaneamine in 0.1 M sodium borate pH 8.5. Use within 1 hour of preparation.</td>
</tr>
<tr>
<td>Cysteine/NaCl</td>
<td>50 mM cysteine and 1 M NaCl in 0.1 M sodium acetate pH 4.3</td>
</tr>
</tbody>
</table>

Recommended immobilization protocol

<table>
<thead>
<tr>
<th>Injection</th>
<th>Flow rate</th>
<th>Contact time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. EDC/NHS (activate the surface)</td>
<td>10 µl/min</td>
<td>2 min</td>
</tr>
<tr>
<td>2. PDEA (introduce reactive disulfide groups)</td>
<td>10 µl/min</td>
<td>4 min</td>
</tr>
<tr>
<td>3. Ligand</td>
<td>10 µl/min</td>
<td>7 min</td>
</tr>
<tr>
<td>4. Cysteine-NaCl (deactivate excess reactive groups)</td>
<td>5 µl/min</td>
<td>4 min</td>
</tr>
</tbody>
</table>
5.2.5 **Aldehyde coupling**

The chemistry of aldehyde coupling is described in Section 4.2.3. The ligand may need to be modified first by oxidation with e.g. sodium metaperiodate to convert cis-diols into aldehyde groups. Procedures for oxidation are described in the literature and are summarized in the Biacore Sensor Surface Handbook.

**Required solutions**

EDC and NHS are included in Amine Coupling Kit from GE Healthcare. PDEA is available from GE Healthcare.

<table>
<thead>
<tr>
<th>Required solution</th>
<th>Concentration/Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDC</td>
<td>0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in water</td>
</tr>
<tr>
<td>NHS</td>
<td>0.1 M N-hydroxysuccinimide in water</td>
</tr>
<tr>
<td>Hydrazine* or carbohydrazide</td>
<td>5 mM hydrazine or carbohydrazide in water</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>1 M ethanolamine-HCl pH 8.5</td>
</tr>
<tr>
<td>Cyanoborohydride</td>
<td>0.1 M sodium cyanoborohydride in 0.1 M sodium acetate pH 4.0</td>
</tr>
</tbody>
</table>

*Warning

Hydrazine is extremely toxic. Carbohydrazide is recommended as an alternative reagent.

**Recommended immobilization protocol**

<table>
<thead>
<tr>
<th>Injection</th>
<th>Flow rate</th>
<th>Contact time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. EDC/NHS (activate the surface)</td>
<td>10 µl/min</td>
<td>3 min</td>
</tr>
<tr>
<td>2. Hydrazine or carbohydrazide (introduce hydrazide groups)</td>
<td>10 µl/min</td>
<td>7 min</td>
</tr>
<tr>
<td>3. Ethanolamine (deactivate excess reactive groups)</td>
<td>10 µl/min</td>
<td>7 min</td>
</tr>
<tr>
<td>4. Ligand (immobilize the ligand)</td>
<td>10 µl/min</td>
<td>7 min</td>
</tr>
<tr>
<td>5. Cyanoborohydride (stabilize the bond)</td>
<td>2 µl/min</td>
<td>20 min</td>
</tr>
</tbody>
</table>
5.2.6 Results of immobilization

Judge the results of immobilization in terms of the amount of ligand remaining on the surface at the end of the immobilization procedure, measured as the response relative to the baseline before surface activation. Measure the amount of ligand attached after final deactivation of the surface (which also serves to remove non-covalently bound ligand). The amount bound before the deactivation step can provide useful information for troubleshooting purposes (see Section 5.4).

Figure 5-1. Sensorgram from a typical amine coupling, illustrating the distinction between the amount of ligand bound and the amount immobilized.

Surface activation itself results in a small change in response (of the order of 100-200 RU for activation with EDC/NHS), but this may be ignored in measuring the amount of ligand immobilized. The correction is small in relation to the high levels of ligand generally recommended for concentration analysis, and a more relevant measure of the level of immobilization is the capacity of the surface for binding analyte.

When the ligand is a small molecule, the response that derives from immobilized ligand is low, and the results of immobilization can only be assessed accurately in terms of the analyte binding capacity of the surface.

5.3 Testing the surface

Once the surface has been prepared, the analyte binding activity should be tested before proceeding to further stages in assay development. The same protocol can also be used to test the activity of sensor chips that have been stored, to ensure that activity is retained throughout the storage.
To test the binding activity of the surface, inject analyte that is known to bind to the ligand. Use successively higher analyte concentrations (e.g. 10, 100, 500, 1000 nM) with a moderate flow rate. The response reached in a single injection can be used to check the consistency of surface activity between different surfaces and during storage of surfaces.

To estimate the maximum analyte binding capacity of the surface, perform repeated injections of analyte without regenerating the surface between injections. The sensorgram will show a steadily increasing response over the initial baseline for each successive injection. Eventually, injection of analyte will give no further increase in response when the maximum binding capacity is reached. This information can give a valuable indication of the potential operating range of the assay.

![Graph showing repeated injections of analyte without regeneration can be used to estimate the maximum analyte binding capacity of the surface.](image)

**Figure 5-2.** Repeated injections of analyte without regeneration can be used to estimate the maximum analyte binding capacity of the surface.

5.4 Troubleshooting surface preparation

This section deals with the most common problems associated with ligand immobilization.

5.4.1 Low immobilization levels

If the level of immobilized ligand is too low, examine the immobilization results to identify the cause of low immobilization levels:

**If ligand does not bind sufficiently to the surface (Figure 5-3):**

- Test pre-concentration at different pH values. As a general rule, pH values down to about 4.0 can be used. If pre-concentration is inadequate even at pH 4.0, the ligand may be too acidic, and you should consider using a different immobilization approach.

- Make sure you are using low ionic strength buffer. The total ion concentration should ideally be 10 mM or less. Only use higher salt concentrations if this is necessary to maintain ligand stability.
• Increase the contact time if the immobilization sensorgram indicates that more ligand can bind.

• Increase the ligand concentration.

![Sensorgram Diagram]

**Figure 5-3.** Inadequate binding of ligand to the surface is seen as a poor increase in response after the ligand injection (the illustration shows a sensorgram for amine coupling).

**If ligand binds to the surface but is not immobilized (Figure 5-4):**

- Make sure you are using fresh EDC and NHS solutions.

- Make sure that the immobilization buffer and running buffer do not contain substances which compete with the ligand for reactive groups on the surface (e.g. primary amines for amine coupling).

- Use a longer contact time for the ligand (the immobilization reaction may be slow).

- Consider an alternative immobilization chemistry.
5.4.2 Low analyte responses

If the amount of ligand immobilized appears to be sufficient but the analyte response or maximum binding capacity is too low, the ligand may have been inactivated during the preparation or immobilization procedure. Try alternative immobilization methods or use a capturing approach. Review the composition of the immobilization buffer (e.g., avoid chelating agents for proteins that require metal ions for activity).

If both the analyte and ligand are large molecules and the amount of ligand on the surface is high, steric hindrance may reduce the maximum binding capacity of the surface. Use lower levels of immobilization for large molecules.

Figure 5-4. Inadequate immobilization of ligand is seen as a large drop in response as a result of the final washing step (the illustration shows a sensorgram for amine coupling).
Regeneration is the process of removing bound analyte from the sensor chip surface after analysis of a sample, in preparation for the next analysis cycle. The number of times a sensor surface can be regenerated depends on the nature of the attached ligand, but is usually greater than 100 and may even be 1000 or more.

When the ligand is attached directly to the surface, regeneration removes analyte from the ligand without destroying the ligand activity. When a capturing approach is used (Section 4.2.5), regeneration generally removes both ligand and analyte from the capturing molecule. In this case the stability of the ligand under regeneration conditions is irrelevant.

Efficient regeneration is important for successful assays. Incomplete regeneration or loss of the binding activity from the surface will impair the performance of the assay and the useful lifetime of the sensor chip will be shortened. Time spent on establishing suitable regeneration conditions is therefore a valuable investment.

### 6.1 Regeneration scouting strategy

#### 6.1.1 Choice of regeneration solution

The choice of conditions for regeneration is dictated by the nature of the ligand-analyte interaction and the stability of the ligand and analyte (or, if a capturing approach is used, the nature of the capturing molecule-ligand interaction and the stability of the capturing molecule).

Experience at GE Healthcare has shown that while different applications may need individually tailored regeneration conditions, many surfaces can be regenerated using brief exposure to acidic (glycine-HCl buffer or dilute HCl) or basic (NaOH) solutions. A selection of ready-to-use regeneration solutions is available from GE Healthcare.

If the sensor surface is not efficiently regenerated with high or low pH (either because the analyte is not fully removed or because the ligand loses activity), other conditions that may be tested, alone or in combination with high or low pH, include:

- high ionic strength (e.g. 1–2 M NaCl or 1–4 M MgCl2)
- up to 100% ethylene glycol
- low concentrations of SDS (e.g. 0.05%)
- 20–100 mM NaOH in 30% acetonitrile (particularly useful for regenerating low molecular weight ligands)
Some ligands that are difficult to regenerate using a single injection may respond better to two sequential injections of different regeneration solutions.

Always make sure that the regeneration solution is fully compatible with the running buffer, so that precipitation does not occur at interfaces between the two solutions. Thus 4 M MgCl₂ causes precipitation with phosphate buffers, and SDS may precipitate if the potassium content of the running buffer is too high, or the temperature of the regeneration solution is too low. Use a different running buffer if precipitation occurs on mixing with the regeneration solution.

### 6.1.2 Testing regeneration conditions

Start investigating regeneration using mild conditions, and then increase the harshness of the treatment progressively until suitable conditions are found. In this way, you minimize the risk of damaging the ligand or capturing molecule.

Testing regeneration conditions involves injection of analyte over the surface followed by regeneration solution. The extent to which the analyte is removed is indicated by the response after the regeneration injection. A further injection of analyte is necessary to test whether the ligand is still active (see Figure 6-1).

![Figure 6-1. Efficient regeneration removes all bound analyte. Inefficient regeneration leaves analyte on the surface (top panel). A second injection of analyte reveals whether the ligand is still fully active (bottom panel).](image)

**Note:** Injection of regeneration solution often gives a considerable bulk response, since the refractive index is not matched with the running buffer. The relative bulk response may be either positive or negative depending on the solution used.
For a new ligand, you may want to perform exploratory tests with single analyte injections to establish whether a particular regeneration strategy is worth pursuing. However, to establish regeneration conditions reliably it is necessary to perform a series of repeated cycles of analyte injection and regeneration, in order to reveal trends in regeneration efficiency and ligand activity. Two to five cycles of analyte binding and regeneration are recommended in testing new regeneration conditions. Once conditions have been found, more extensive tests may be performed to establish that the assay is stable through large numbers of repeated cycles (see Section 7.7).

In general, use short contact times (typically 30-60 seconds) for regeneration. Longer exposure to regeneration conditions wastes time and involves greater risks for loss of ligand activity.

### 6.2 Interpreting regeneration scouting

#### 6.2.1 Report point placing

Two parameters are important for assessing regeneration: the consistency of analyte response (representing the activity of the surface) and the baseline level (representing the amount of material remaining on the surface). These are measured by placing report points shortly before the beginning and after the end of the sample injection (for baseline and analyte response respectively).

Because of the way analysis cycles are constructed, the report points in any given cycle are placed before the regeneration attempt in the cycle. In consequence, the effect of the regeneration attempt in one cycle is seen from the report points in the next cycle (Figure 6-1).

#### 6.2.2 Trend plots

To assess the efficiency of regeneration, prepare trend plots of the baseline level and analyte response as a function of cycle number. Group the points according to the conditions tested. Remember that the results in cycle 2 show the efficiency of regeneration in cycle 1, so that if each condition is tested with five replicate cycles, the points should be grouped as cycle 1, cycle 2-6, cycle 7-11 and so on.

Each group in a plot of this kind shows the trends in baseline and analyte response within replicate cycles of the same regeneration conditions. The trends between groups show the effect of changes in conditions.

The following guidelines may help in assessing regeneration:

- The sample binding response should be constant. A falling trend in sample response indicates either that the ligand is losing activity (regeneration is
too harsh) or that material is accumulating on the surface (regeneration is too mild).

- The baseline after regeneration should not increase. If it does, this indicates that material is accumulating on the surface (regeneration is too mild). Some increase in baseline may however be tolerated provided that the analyte binding response is not affected.

- The baseline level after regeneration may fall, particularly during the first few cycles using a newly prepared chip. This is acceptable provided that the analyte response is not affected. However, if both baseline and analyte response show a falling trend, ligand is probably being lost from the surface as a result of excessively harsh regeneration.

![Figure 6-2. Scouting for regeneration conditions. The report points from the first cycle give the starting values: thereafter the points are grouped according to the regeneration conditions tested. See text for interpretation. • = baseline response; x = sample response.](image)

In the example shown above, the baseline increases from the starting value and stays high during the first regeneration condition tested (A). Conversely, the sample response falls and stays low, suggesting that analyte is not removed from the surface: the first conditions are too mild. The second condition tested (B) shows some improvement, while the third (C) results in a progressively decreasing baseline with maintained (or even slightly improved) sample response. Condition D results in deterioration of the sample response. However, none of the conditions illustrated is ideal, and optimization of conditions intermediate between C and D could result in further improvement.

It is crucially important in concentration measurements that the response obtained from repeated cycles using the same sample is constant. If the response falls during the course of an assay, as a result of either incomplete
regeneration or inactivation of the ligand through excessively harsh conditions, this will introduce an unacceptable dependence of the assay calibration on cycle number. If fully acceptable regeneration conditions cannot be established, the choice of ligand and/or assay format should be reviewed. In some cases a capturing approach can be advantageous since ligand is replenished in each cycle, and inactivation of the ligand by regeneration solution is not a concern. In a capturing approach, however, the considerations for ligand regeneration discussed in this chapter apply to regeneration of the capturing surface.
6 Regeneration
6.2 Interpreting regeneration scouting
Developing and running concentration assays

Chapters 4–6 have dealt with surface preparation and regeneration, which are essential components of development of any application for Biacore. This chapter considers further aspects that apply to concentration assays. The chapter covers:

- Assay requirements
- General practical considerations
- Adjusting the operating range
- Non-specific or unwanted binding
- Specificity
- Matrix interference
- Assay stability
- Assay development for calibration-free assays

Although aspects of assay development are considered in separate sections in this chapter and are generally approached with separate experimental design in practice, the process of development should result in an assay that meets all requirements at the same time. In many cases, compromises between different requirements may be necessary. For example, a detecting molecule that gives an assay with an excellent range may be unsatisfactory in terms of specificity, while an alternative molecule that has acceptable specificity may be inferior in terms of range. Development of an assay is an iterative process of refinement, and it is often most useful to test all requirement aspects roughly before proceeding to optimize each aspect. In that way, the risk is reduced that work put in to optimizing one aspect may be wasted when another aspect proves unsatisfactory.

7.1 Assay requirement specification

As a prelude to development of a concentration assay, an assay requirement specification should be established, detailing the demands that the assay is required to meet. The goal of assay development is then to meet the requirement specification.

Parameters describing assay performance are explained in Section 2.2. In general, an assay requirement specification should define, in as much detail as possible, the acceptable range of values for each of the performance parameters that is relevant to the assay in question.
7.2 General practical considerations

7.2.1 Preparing samples

Concentration assays using Biacore rely on specific interaction between the analyte and detecting molecule, and the SPR detection technology (see Appendix A) allows measurements to be made on colored or turbid (even opaque) samples. In consequence, sample preparation is often greatly simplified in comparison with many other established techniques for concentration determination.

Bear in mind the following points with respect to sample preparation:

- Although measurements can be made on turbid and opaque samples, repeated injection of particulate suspensions can lead to problems with the flow system in Biacore. Samples should be centrifuged (e.g. 15,000 g for 10 minutes) or filtered (0.22 µm filter) if possible before analysis.

- Some kind of extraction procedure is necessary for measurements on solid or particulate matrices. If extraction is performed using organic solvents, extracts will need to be transferred to water-based media before analysis. Refer to the chemical resistance information for the Biacore system for compatibility of the flow system and sensor surface with organic solvents.

- Partial purification or fractionation of samples or extracts may be necessary to eliminate non-specific binding or matrix interference effects (Section 7.6). Use standard fractionation procedures appropriate for the analyte being measured.

In Biacore systems that do not have a built-in degasser, both running buffer and buffers used in preparation of samples should be thoroughly degassed to avoid formation of air bubbles in the flow system during the run.

Remember that samples may need to be diluted so that the concentration falls within the measuring range of the assay. In general, samples should be diluted in running buffer.

7.2.2 Report point placing

The response observed during the sample injection in Biacore results from a combination of analyte binding to the sensor surface and differences in bulk refractive index between the sample and running buffer (Figure 7-1).
Developing and running concentration assays

Figure 7-1. The observed response results from a combination of analyte binding to the sensor surface and differences in bulk refractive index between the sample and running buffer.

Careful matching of the buffer composition between sample and running buffer can help to reduce the bulk contribution, but exact matching is seldom possible for measurements made on complex sample matrices. Where possible, place report points for measuring analyte response shortly (5-30 seconds depending on the rate at which analyte dissociates) after the sample injection, to avoid the bulk refractive index contribution from the sample (Figure 7-1). Placing of the report point before the end of the injection is only necessary for analytes that dissociate rapidly from the surface, making reliable measurements after the sample injection more difficult: in such cases, the refractive index of sample and running buffer should be carefully matched.

Figure 7-2. Place report points for measuring response after the sample injection so that the measured response is not affected by the bulk refractive index of the sample.

Binding rates measured from the slope at a report point during sample injection are not affected by bulk refractive index considerations. Report points for binding rate measurements should be placed so that the window for slope determination is clear of any bulk refractive index contribution or other disturbances at the beginning of the sample injection.
Always place report points at identical positions on all sensorgrams for both calibration and unknown samples. If any of the report point values is invalidated by temporary disturbances in the sensorgram (such as air bubbles on the sensor surface), either eliminate that particular cycle from the evaluation or assign a different report point in all cycles. Do not move report points in some cycles but not in others.

Calibration-free concentration measurements are based on fitting the sensorgrams to a mathematical model of the interaction (Appendix B), and do not use report points. Bulk refractive index changes are included in the fitting model and generally do not interfere with the measurements.

7.3 Adjusting the operating range

The operating range of an interaction-based assay is defined in terms of lower and upper limits that can be measured with acceptable performance. The range cannot extend at the lower limit beyond the limit of quantitation (LLOQ) for the assay, which is a combined measure of the observed response in relation to the noise level of the detection system and the experimental variations in sample preparation and measurement (see Section 2.2.5). The upper limit (ULOQ) is set by the characteristics of the assay format that determine the shape of the calibration curve. Samples with concentrations above the upper limit of the operating range cannot be measured without prior dilution.

The main factors influencing the operating range of a calibrated assay are the affinity of the detecting molecule for the analyte, the concentration of detecting molecule and the contact time of the sample with the sensor surface. The ways in which the operating range can be adjusted differ according to the format of the assay.

7.3.1 Direct binding assays

The typical shape of the calibration curve for a direct binding assay is shown in Figure 7-3. The lower limit of the operating range is set by the noise characteristics of the assay at low response levels. The upper limit is set by the shape and position of the standard curve in relation to analyte concentrations, since the sensitivity of the assay decreases with increasing concentration.

- The affinity of ligand for analyte affects the position of the curve on the concentration axis. As an approximate guideline, half-maximum response is obtained when the analyte concentration is the same as the affinity constant for the interaction (K_D) if the interaction is allowed to proceed to equilibrium (this is precisely true for 1:1 interactions and can be taken as a first approximation for most interaction-based assays).
Figure 7-3. Using a ligand with a higher affinity moves the calibration curve towards lower analyte concentrations.

- The amount of immobilized ligand affects the height of the curve on the response axis: a higher level of ligand gives a higher maximum analyte response. Higher response levels will give greater precision in the measurements so that the range of the assay is extended but not shifted.

Figure 7-4. Higher amounts of immobilized ligand increase the response but have little effect on the shape of the calibration curve.

- The contact time for the sample affects both the height and position of the calibration curve. If the response is measured early during the association phase, the height will be reduced and the curve will be shifted towards higher concentrations.
7 Developing and running concentration assays
7.3 Adjusting the operating range

Enhancement reagents
For assays using enhancement reagents, additional factors influencing the range are the affinity, concentration and contact time for the enhancement reagent. In general, enhancement reagents should be used at relatively high concentrations (one to two orders of magnitude higher than the affinity constant, i.e. typically 10-100 µg/ml to ensure rapid and complete binding with short contact times).

To optimize the range of a direct binding assay, determine a calibration curve to establish the approximate range of the assay and then use the principles described above to make appropriate adjustments. If the range requirements cannot be met by adjusting the contact time or ligand level, you will need to review your choice of ligand and possibly your choice of assay format.

7.3.2 Inhibition assays
The calibration curve for an inhibition assay is inverted in comparison with a direct assay, with the highest response at low analyte concentrations.

- The affinity of the detecting molecule for the analyte in solution together with the concentration of detecting molecule determine the useful range of an inhibition assay. Higher affinities allow measurement at lower analyte concentrations but also result in a narrower operating range (Figure 7-6). The relationship between affinity and range is more complex than for direct assays since the detecting molecule binds to analyte both on the surface and in solution, but the principle is similar.
Developing and running concentration assays

Figure 7-6. Increasing the affinity of the detecting molecule moves the operating range to lower analyte concentrations and also narrows the range. Increasing the concentration of detecting molecule moves the operating range to higher concentrations.

- The concentration of detecting molecule affects the height of the curve on the response axis. Higher concentrations will compress the curve at high response levels, while lower concentrations will reduce the maximum response and potentially allow measurement at lower analyte concentrations.

Figure 7-7. Reducing the concentration of detecting molecule reduces the maximum response but may allow measurement at lower analyte concentrations.

- The contact time for the sample affects both the height and position of the calibration curve in the same way as for direct assays (see Section 7.3.1). Use a longer contact time to increase the response range. Combine a
7 Developing and running concentration assays
7.3 Adjusting the operating range

Follow the steps below to find the best calibration curve that can be obtained for a given detecting molecule. The sensor surface must be prepared first by immobilization of analyte or analyte analogue.

1. Inject the detecting molecule at a range of concentrations with no added analyte. Select the concentration that gives a suitable response level (approximately 100-2000 RU is recommended depending on the performance requirements) within a reasonably short contact time.

2. Prepare a calibration curve using the selected concentration of detecting molecule with added analyte over at least the required operating range of the assay.

3. If the calibration curve does not exploit the full response range (i.e. the highest concentration of analyte does not reduce the response to baseline), repeat the calibration curve using a lower concentration of detecting molecule and a longer contact time to compensate for the reduced maximum response. It may sometimes not be possible to achieve complete inhibition.

4. If you cannot meet the required assay specification by adjusting the concentration of detecting molecule and contact time for the injection, the affinity of the detecting molecule for analyte is probably too low. Review your choice of detecting molecule. In comparing calibration curves obtained with different detecting molecules, adjust the respective concentrations so that the maximum response level is comparable between different detecting molecules.

7.3.3 Calibration-free concentration assays
For calibration-free measurements (based on determination of binding rates at two or more different flow rates, see Section 3.3), the useful range of the assay is determined by the range of binding rates that can be reliably analyzed by the fitting procedure. As a rule of thumb, the initial binding rate should be in the range 0.3–15 RU/s at a flow rate of 5 µl/min, and the sensorgram should be approximately linear over the first 36 seconds or more of the interaction. This range of measured binding rates corresponds to a concentration range of 0.05–5 µg/ml. If the original concentration is higher than this range, the samples should be diluted. In most cases, measuring a series of 10-fold dilutions of the sample will cover sufficient range for determination of the concentration in unknown samples, provided that the original concentration is not below the useful range of the assay.
Note: Calibration-free assays are independent of the interaction characteristics between ligand and analyte, since the determination is based on measuring the binding rate under conditions where the rate is limited by analyte diffusion properties.

7.4 Unwanted binding of analyte or detecting molecule

Unwanted binding refers to binding of analyte (or detecting or competing molecule in indirect assays) to the carboxymethylated dextran matrix on the sensor surface. Non-specific binding of components from the sample matrix is considered in Section 7.6.1.

7.4.1 In direct binding assays

Analyte that binds to the dextran matrix on the sensor surface in a direct assay will contribute to the measured response in the same way as analyte that binds to the immobilized ligand. Efficient regeneration has sometimes proved difficult where macromolecular analytes bind directly to the dextran matrix. Addressing the unwanted binding issue can improve assay performance and in some cases help to simplify optimization of regeneration conditions.

7.4.2 In inhibition and surface competition assays

If analyte binds to the dextran matrix in indirect assay formats, it is likely that the efficiency of the competitive situation will be reduced. In inhibition assays, it is essential that the complex of analyte and detecting molecule in solution is unable to bind to the surface, and this will probably not hold true if the analyte can bind directly to the dextran matrix. Surface competition assays rely on a well-defined competition between analyte and competing molecule for a common binding site on the surface, and binding to the dextran matrix will usually obscure this competition. The same arguments hold for binding of detecting molecule or competing molecule to the dextran matrix.

The effect of this kind of unwanted binding is to reduce the response range of the assay and (depending on the characteristics of the non-specific binding) potentially impair the operating range.
7 Developing and running concentration assays
7.4 Unwanted binding of analyte or detecting molecule

Figure 7-8. Unwanted binding of either analyte or detecting molecule to the dextran matrix in an inhibition assay will introduce a background response level, reducing the response range of the assay.

7.4.3 In calibration-free assays
Unwanted binding in calibration-free concentration assays will interfere with the determination if the binding shows a measurable rate during the sample injection phase. Binding that is very rapid will be seen as a bulk refractive index shift by the evaluation procedure and will not affect the measurements provided that the binding rate after the initial shift can be evaluated adequately.

7.4.4 Testing for unwanted binding
To test for binding of analyte or detecting molecule to the dextran on the sensor surface, inject samples over an unmodified surface. If binding is negligible, the issue is not a problem. Note however that significant binding to a blank unmodified surface does not mean that analyte will bind to the surface in assay situations. The immobilization chemistry used to attach ligand to the surface generally reduces the charge density on the dextran, reducing the tendency for electrostatic binding, and the high density of ligand recommended for concentration measurements can help to reduce binding of analyte to the dextran.

In testing for unwanted binding, always assess the observed levels of binding in comparison to the range of response values expected in assay situations. Low levels of unwanted binding may be tolerated if the assay performance is not significantly affected.
7.4.5 Dealing with unwanted binding

The following steps can help to reduce unwanted binding:

- Modification of the assay buffer can in some cases help to reduce binding to the dextran matrix. In general, physiological salt concentrations (0.15 M) or higher should be used to reduce electrostatic binding to the surface. The buffer substance used can also influence the binding of analyte to the dextran matrix.

- Different sensor chip surfaces are available from GE Healthcare. If binding to the dextran matrix is a problem, try using a sensor chip with a different surface matrix.

- If unwanted binding in indirect assays cannot be reduced or controlled sufficiently by optimizing assay conditions, you may need to review your choice of detecting or competing molecule.

7.5 Specificity and cross-reactivity

The specificity of a concentration assay is defined in relation to detection of known compounds related to the analyte. To determine specificity, measure calibration curves for the respective compounds separately under the same conditions.

7.5.1 Measuring specificity

Measure the intended analyte over the required concentration range for the assay. Measure other compounds over a wider concentration range (in particular at higher concentrations) to enable detection of low levels of cross-reactivity. As a general guide, determine the analyte concentration that gives 50% of maximum response (commonly termed B$_{50}$ in direct binding contexts and IC$_{50}$ for inhibition assays), and use concentrations of other substances up to 100 times this value. This will enable detection of cross-reactivity down to levels of 1%. Make sure that the flow system is washed thoroughly between cycles to eliminate carry-over from high concentration samples. In determining cross-reactivity for analytes of different molecular weight in a direct assay, divide the observed response by the molecular weight to correct for the differences in size.

Quantitatively, cross-reactivity may be expressed in terms of the ratio of concentrations that give the same response for the analyte and the compound in question, after adjustment of the response for analyte molecular weight if necessary (see Section 2.2.1). A compound that requires a 100 times higher concentration to achieve the same response as the analyte is said to show 1% cross-reactivity.
7.5.2 Optimizing specificity

As a general rule, the target for cross-reactivity in assay development is either "all-or-nothing". For assays designed to measure the concentration of a class of related compounds (for example different metabolic forms of vitamins), cross-reactivity with the analyte should be as high as possible. Compounds that should not be included in the measured concentration should conversely show cross-reactivity close to zero.

Choice of detecting molecule and/or assay format is the most useful tools in optimizing assay specificity. If the specificity is not satisfactory, adjustment of the buffer conditions may in some cases help to improve the results. There are no general rules for this kind of optimization, since effects of buffer conditions will vary for different analytes and related compounds.

- A direct assay which shows broad specificity in the sample response may in many cases be improved by the use of an enhancement reagent, since the analyte is then identified by simultaneous binding to two different molecules. This is generally most useful for macromolecular analytes.
• Monoclonal antibodies used as ligand or detecting molecule generally provide a higher specificity than polyclonal antibodies. When a panel of monoclonal antibodies is available, the specificity may vary significantly between different antibodies.

7.5.3 Specificity in calibration-free assays

In calibration-free assays, any components in the sample that interact with the surface with a measurable binding rate will contribute to the calculated concentration. The results are however evaluated on the basis of a diffusion constant for a single molecular species. In consequence:

• Calibration-free assays will correctly measure the total concentration of all interacting molecular species provided that the physical characteristics of the species are sufficiently similar. For example, a calibration-free measurement will determine the total specific antibody concentration in a polyclonal antibody preparation of IgG containing multiple subclasses, since all IgG molecules have essentially the same molecular weight and diffusion characteristics.

• On the other hand, calibration-free assays cannot measure the true concentration in a mixture of differently-sized molecules that interact with the ligand. For example, measurements on a mixture of IgG, IgE and IgM antibodies will not give a correct concentration since the different isotypes vary significantly in molecular weight. In cases like this, the concentration obtained is that which would apply to a homogeneous preparation of molecules with the specified diffusion coefficient.

7.6 Matrix interference

Matrix interference refers to the effects that non-analyte components in the sample matrix can have on the performance of the assay. This becomes an important issue for assays that are designed to be used in different sample matrices. Generally, separate calibration curves will be needed for each sample matrix, although in some cases (such as individual serum samples) matrix variation cannot be avoided. Matrix interference can however also be relevant in single-matrix assays where the calibration curve and samples are measured in the same environment, since interfering factors can impair the assay performance.

Matrix interference may be broadly divided into two aspects:

• Non-specific binding, i.e. binding of non-analyte components in the sample to the sensor surface.
• Interference from matrix components in the binding of analyte to the detecting molecule.
7.6 Matrix interference

7.6.1 Non-specific binding to the sensor surface

Effects of non-specific binding

Non-analyte components that bind to the surface in direct assays or to the detecting molecule in inhibition assays will contribute to the response in the same way as analyte, and will not in fact be distinguishable from analyte in the results of the assay unless an enhancement reagent is used to confirm analyte identity.

An additional form of non-specific binding can arise in inhibition assays, from matrix components that bind to analyte on the sensor surface and thus generate response in the same way as the detecting molecule. This kind of interference will lead to underestimation of the analyte concentration.

Testing for non-specific binding

Test for non-specific binding by injecting negative sample matrices, free from analyte, over the surface. A representative set of the matrices that will be used in the final assay situation should be tested. In cases where the sample matrices are subject to individual variations (e.g. patient serum in clinical assays), the question can be addressed either by screening a randomly selected group of negative sample matrices or by pooling a group of matrices and testing the pooled material. In either case, however, the possibility remains that an individual sample will show non-specific binding that deviates from the control group.

For inhibition assays, test sample matrices with and without addition of detecting molecule and compare the response with that obtained with detecting molecule alone, to reveal interfering components that mimic the analyte and detecting molecule respectively.

Dealing with non-specific binding

Ideally, the goal of assay development should be to eliminate all binding of non-analyte components, so that the assay is entirely specific for analyte. This is however not always feasible, and the approach to dealing with non-specific binding of this kind depends largely on the situations in which the assay will be used. If analyte concentrations are always measured in a well-defined sample matrix, the calibration curve can be prepared in the same matrix, and the effect of non-specific binding will be controlled within the assay framework. For assays designed to be used in a range of different sample matrices, the following measures can help to reduce non-specific binding:

- Enhancement reagents can be used in direct binding assays to improve specificity. Non-analyte components in the sample that bind to the surface will not be detected by the enhancement reagent if the reagent is chosen carefully.
Developing and running concentration assays

7.6.2 Other matrix interference effects

Other possible sources of matrix interference include:

- Sample components that bind to analyte may result in either under- or overestimation of the analyte concentration. If the analyte is made unavailable to the detecting molecule, the concentration will be underestimated. Binding that increases the effective mass of the analyte without affecting the interaction with the detecting molecule will however result in overestimation of the concentration in direct assays but will have no effect in inhibition assays.

- Sample components that interfere with the analyte binding to the detecting molecule can affect the assay performance in a number of ways. For example, inhibitors of the interaction will reduce the apparent analyte concentration, while cofactors that promote the interaction may affect the affinity and therefore the operating range.

- In indirect assays, binding of large components to an independent site on the detecting molecule will increase the size of the detecting molecule, giving a higher response that results in underestimation of the analyte concentration.

- Components that modify the analyte may change the binding properties resulting in either over- or underestimation of the concentration. Such changes may occur during the course of the assay, resulting in progressively increasing matrix effects. One example is protease activity in the sample that gradually destroys the analyte.

Detecting matrix interference

To check for matrix interference, prepare a calibration curve using analyte in buffer, and determine the recovery of analyte (expressed as measured value divided by expected value) for at least two analyte concentrations in a dilution series of the sample matrix from the maximum matrix concentration down to 0%. Matrix interference is revealed as a variation in recovery values as a function of matrix concentration.
In addition, run analyte recovery determinations in sample matrix over a time period at least as long as the maximum expected duration of an assay, to test for any time-dependent matrix effects.

As a final control for the absence of matrix interference, check for interference using analyte concentrations at the extremes of the usable range, to establish whether interference has been eliminated over the whole intended range of the assay.

**Dealing with matrix interference**

The following measures can help to reduce or eliminate matrix interference:

- Dilute the samples to reduce the matrix concentration. This measure may or may not be successful depending on the type of interference: the tests performed to reveal matrix interference should indicate whether dilution is a useful option. You may have to adjust other aspects of the assay to compensate for the lower analyte concentrations in the diluted samples.

- Test the effect of using different sample buffers.

- Refine the sample preparation steps to eliminate the interfering component(s).

- As a last resort, review your choice of detecting molecule and/or assay format.

### 7.7 Assay stability

#### 7.7.1 Testing assay stability

As a final step in assay development, you should check that performance is maintained over the maximum intended number of samples for the assay. Run replicate samples of the same known analyte concentration throughout. Ideally, the results should be constant throughout the assay. Check the results for trends in sample response and for the consistency of calibration curves from different times in the assay.

#### 7.7.2 Dealing with assay stability issues

If the performance varies to an unacceptable degree during the course of the assay, check the following possibilities:

- Regeneration problems. If regeneration is not fully optimized, the baseline and analyte binding capacity of the surface may be progressively affected. See Chapter 6 for considerations relating to regeneration.

- Evaporation from samples. If the samples are not properly sealed, evaporation during the course of the assay will result in steadily increasing
concentrations. The effect of this will differ according to whether evaporation affects the calibration samples or the test samples, and all or only a few samples. For Biacore-based assays, vials should be securely capped and microplates should be sealed with adhesive foil. For both caps and foil, use only materials supplied or recommended by GE Healthcare, to avoid problems with the sample dispensing mechanism.

- Either the analyte or the detecting molecule may be inherently unstable under the conditions of the assay. Optimizing buffer conditions may help to improve stability: inclusion of moderate concentrations of sucrose (up to 10%) or glycerol (up to 20%) can stabilize proteins without adversely affecting the assay characteristics. Bear in mind however that inclusion of sucrose or glycerol in the samples will introduce a considerable bulk refractive index contribution.

Stability issues that cannot be satisfactorily solved may limit the number of cycles for which an assay can be used.

7.8 Running routine assays

Once the assay conditions have been determined so that the performance requirements are met, the assay can be established as a routine procedure. Biacore systems designed for use in GxP environments include software functionality that allows the assay developer to “publish” procedures for routine use. Assay settings determined during development are locked in published procedures, ensuring consistent execution of the assay in routine use.

Bear the following points in mind when setting up and running routine assays:

- Always include control samples with known analyte concentration at regular intervals through the assay, to confirm that the assay performance remains constant within acceptable limits.

- If performance drift is known to be a problem, run calibration curves using standard analyte samples at the beginning and end of the assay run, and at suitable intervals during the assay. Samples can be evaluated in relation to the closest calibration curve, reducing the effect of the drift.

- Document all aspects of the assay thoroughly, including sample preparation, assay operation and evaluation. Indicate clearly which procedural settings can be changed by the user without compromising the performance of the assay.

- Prepare samples as reproducibly as possible and in accordance with the assay documentation.
7 Developing and running concentration assays
7.8 Running routine assays
8 Measuring concentration in GxP environments

This chapter provides guidelines for setting up, validating and running assays in GxP environments.

Demands for working under GxP regulations may be set by external authorities, company policy or individual laboratory practice. Even at the external authority level, there is currently no agreed standard that defines GxP requirements, although guidelines as published by ICH (see reference on page 11) are accepted in many cases. Interpretation and implementation of the guidelines is the responsibility of the individual company or laboratory. Recommendations in this handbook are based largely on the ICH guidelines.

In most regulated assay environments, there will be a distinction between assay developers, responsible for developing and optimizing assay conditions, and routine operators who perform the concentration measurements on “real” samples. Normally, only the routine operation environment needs to be regulated: however, it is an advantage in most situations (and may be a demand in some cases) that the development work is carried out as far as possible in a regulated environment. If development is performed according to GxP guidelines, it will be much simpler to establish the routine assay as a validated procedure.

8.1 Setting up GxP assays

8.1.1 System performance

System performance is a validation parameter that ensures that both assay methodology and equipment perform in accordance with expectation. At a basic level, this is a question of using reliable measuring equipment and established methodology. For strict GxP compliance, however, demands are often made on qualification of equipment and reagents. Equipment qualification procedures may cover documented development and manufacturing procedures as well as regular maintenance and performance testing.

GE Healthcare’s SPR technology is established through wide experience in a large number of independent laboratories and is generally well suited for use in regulated environments. Some systems provide design-validated software and equipment qualification services specifically intended to support work in GxP contexts.
8 Measuring concentration in GxP environments
8.1 Setting up GxP assays

8.1.2 Assay settings
As far as possible, experimental settings for assay procedures used in regulated environments should be fixed (with the obvious exception of sample definition tables), so that the consistency of routine assay execution is ensured. This will contribute significantly to the precision of the assay.

To this end, the control software for Biacore systems designed for concentration measurement includes facilities for locking assay settings by “publishing” assay procedures, such that routine operators cannot access the majority of the settings.

Adequate version management should be implemented for both development and routine assays. Since development is a relatively complex process, it is important to maintain version documentation for the procedures used during development, particularly if more than one person is involved in the process. When an assay is established for routine use, version management is important to keep track of possible revisions of the assay procedure. The assay publishing function in Biacore systems designed for concentration measurement maintains a history of published versions and only permits the most recently authorized version to be used by routine operators.

8.1.3 Assay procedure documentation
The assay procedure should be documented in its entirety, including all steps from sample collection and preparation to evaluation of the results. In cases where the assay settings cannot be locked, the documentation should clearly indicate which settings may be varied by the routine operator.

In Biacore systems, every result file contains a complete documentation of the conditions of the assay; in addition, assay settings may be saved as templates independent of result files. Although this provides adequate documentation to satisfy the demands of many regulated environments, archiving of hard-copy printouts of the settings for routine assays is strongly recommended.

8.1.4 Data storage
Establish a defined structure for storing electronic files associated with routine assays, and if possible use the operating system facilities to restrict the access of developers and routine operators to the relevant parts of the file structure. This will help to maintain structured documentation of assay procedures and results and will assist in issues of traceability. Biacore systems designed for concentration measurement automatically create and use a defined folder structure for file storage.

Make sure that the files and folders related to the assay procedure are backed up on a regular basis.
8.2 Validating GxP assays

This section considers practical aspects of measuring the performance parameters commonly required for assay validation. The parameters are defined in Chapter 2. Where appropriate, determinations should be performed in multiple replicates to provide a statistically significant value for the performance parameters. Variance in parameter values is generally expressed as %CV, which is defined as the standard deviation of the determinations as a percentage of the mean value.

8.2.1 Specificity

Test the specificity of an assay by determining calibration curves for the analyte and for potentially cross-reacting compounds. The choice of compounds to be tested depends on the nature of the analyte and the type of sample matrix in which the assay will be used. The following should be considered as potential sources for cross-reacting compounds:

- Compounds that are chemically or metabolically related to the analyte (e.g. tetrahydrofolic acid is a reduced form of folic acid that may be expected to occur in natural sources).
- Compounds that may be suspected of binding to the detecting molecule even if they are not related to the analyte (e.g. several different and distinct drugs may bind to the same target molecule).
- Modified forms of the analyte (e.g. denatured or aggregated forms, digestion fragments etc). These can often be generated by stressing samples of the analyte in various ways such as heating, oxidation, pH changes etc. In cases where 100% cross-reactivity is desirable, recovery values as described in Section 8.2.2 provide an alternative to calibration curves as a measure of specificity.

8.2.2 Accuracy

Determine accuracy by measurement of reference standards where these are available. Determine recovery of analyte from sample matrices by measuring samples spiked with known amounts of analyte. Measure accuracy and recovery over a range of spiked concentrations that exceeds the required operating range of the assay. Accuracy determinations then form one of the defining parameters for the range of the assay.

8.2.3 Precision

Repeatability (intra-assay precision) is determined as the variance in measured values of replicate samples within one assay. Repeatability is necessarily restricted to one assay instrument and one operator, and may be determined for pure analyte or sample matrices.
Intermediate precision is determined as the variance in measured values of replicate samples between different operators and on different occasions within the same laboratory. Where appropriate, the intermediate precision may be determined separately for different assay instruments.

Reproducibility is determined as the variance in measured values of samples between different laboratories using the same assay on equivalent samples. Normally, this will involve different operators and assay instruments. To avoid inadvertent variation in samples, reproducibility is normally determined using standardized samples of analyte.

The scope of assay precision should be clearly indicated in the performance documentation. In Biacore-based assays and in many other assay formats, sample preparation is distinct from the actual concentration measurement procedure, and in some cases the evaluation procedure may also be subject to experimental variation. The precision of the assay as a whole will include all of sample preparation, measurement and evaluation, but it is often valuable in addition to document the precision of each separate operation. This will help to identify the major source of variation in assay results.

Determine precision over a range of analyte concentrations that exceeds the required operating range of the assay. These determinations then form one of the defining parameters for the range of the assay.

### 8.2.4 Linearity

Linearity is measured in terms of the regression coefficient for least squares fitting to the calibration curve, plotted after appropriate mathematical transformation if necessary to produce a linear relationship.

Biacore-based assays often do not provide data that can be analyzed according to a linear relationship, and a four-parameter equation is provided in systems designed for concentration measurement for fitting a calibration curve to the measured values for standard samples. Linearity can then be determined by plotting the measured value against the known sample concentration for standard samples, and determining the regression coefficient for this plot.

### 8.2.5 Range

The range of an assay is set by the limits of acceptable precision and accuracy. Procedures used to determine precision and accuracy thus provide information on the range. To be relevant for practical use, the range should be determined in terms of either the intermediate precision or the reproducibility, depending on the situations in which the assay will be used. The intra-assay precision will frequently be better than the intermediate precision and is not relevant for the range of an assay.
Depending on how the assay procedure is defined, the range may only have a lower limit (LLOQ, see Section 2.2.5). Assay procedures that permit unlimited dilution of sample before measurement do not have an upper limit to the range.

### 8.2.6 Robustness

Robustness is a measure of the capacity of the method to remain unaffected by small deliberate variations in method parameters. Robustness of a Biacore-based assay should always encompass variations in the amount of ligand attached to the sensor chip surface: in this context it is valuable to determine the variance in amount immobilized with a standardized procedure, as a basis for deciding the extent of variation in robustness testing. Additional factors that should be included in robustness tests are common to most assay methods, for example variations in weight and volume measurements used in preparing samples and buffers.

### 8.3 Running GxP assays

#### 8.3.1 Document the run settings

When running concentration assays in a regulated environment, all run settings should be carefully documented for each assay occasion. The extent of this documentation requirement may be reduced if assay settings are locked (see Section 8.1.2), since the number of parameters that can be varied by the operator is reduced. Biacore systems offer a notebook associated with each run where run settings may be documented. The contents of the notebook are saved automatically in the result file.

#### 8.3.2 Data storage

Use a standardized format for naming result files to aid traceability. Result files should be easily identifiable with respect to operator, analyte and sample set. As a recommendation, include the day’s date in the standardized naming format: even if the date of the assay is recorded in the result file and the creation date for the file is recorded in the operating system, an explicit date in the file name can be a considerable help in identifying files.

#### 8.3.3 Audit trails

In a regulated environment, any changes made to results after an assay is performed must be logged in an audit trail to allow reconstruction of the original results if necessary. In Biacore systems designed for concentration measurement, this feature is integrated in the control software. Results of an assay are evaluated automatically and any changes made to the evaluation are visibly recorded in the result file, and the original results can be restored at any time. The original results generated by the assay cannot be altered.
8 Measuring concentration in GxP environments
8.3 Running GxP assays
Appendix A  The SPR detection principle

Biacore exploits the phenomenon of surface plasmon resonance (SPR) to detect and measure analyte. This appendix gives a brief description of the detection principle.

A.1 Surface plasmon resonance

Surface plasmon resonance is a phenomenon that occurs in thin conducting films at an interface between media of different refractive index. In Biacore, the media are the glass of the sensor chip and the sample solution, and the conducting film is the gold layer on the sensor chip surface.

Under conditions of total internal reflection, the light leaks an electric field intensity called an evanescent wave field across the interface into the medium of lower refractive index, without actually losing net energy. The amplitude of the evanescent field wave decreases exponentially with distance from the surface, and the effective penetration depth is about half the wavelength of the incident light.

Figure A-1. The SPR detection principle.
At a certain combination of angle of incidence and energy (wavelength), the incident light excites plasmons (electron charge density waves) in the gold film. As a result, a characteristic absorption of energy via the evanescent wave field occurs and SPR is seen as a drop in the intensity of the reflected light (Figure A-1).

Because the evanescent wave field penetrates the solution, conditions for this resonance effect are very sensitive to the refractive index of the solution within the effective penetration depth of the evanescent field. Changes in solute concentration at the surface of the sensor chip cause changes in the refractive index of the solution which can be measured as changes in the SPR conditions.

**Note:** The reduced intensity of reflected light is not caused by light absorption in the sample in the conventional (transmission spectroscopy) sense. The light used in Biacore is totally internally reflected inside the optical unit, and it is the evanescent wave that penetrates the sample. Consequently, measurements may be made on turbid or even opaque solutions, without interference from conventional light absorption or scattering by the sample.

### A.2 What SPR measures

In the configuration used in Biacore, the SPR response is a measure of the refractive index of the solution within the penetration distance of the evanescent field wave. This distance is small (about 300 µm) in relation to the volume of sample used, so that effectively SPR measures the refractive index at the surface of the sensor chip.

The refractive index of the solution varies with the solute content. When the detecting molecule is attached to the sensor chip or when analyte binds to the detecting molecule, the solute concentration at the sensor chip surface increases leading to a change in the SPR signal. The response measured in Biacore is related to the mass of analyte bound and is largely independent of the nature of the analyte. Refractive index contributions for different solutes are additive, so that the amount of detecting molecule attached and the amount of analyte bound can both be measured with the same detection principle.

### A.3 Biacore configuration

Light from a near-infrared light-emitting diode (LED) is focused on to the sensor chip surface in a wedge-shaped beam, giving a fixed range of incident light angles (Figure A-1). Light reflected from the sensor chip is monitored by a linear array of light-sensitive diodes covering the range of incident light angles. Computer interpolation algorithms determine the angle of minimum reflection (the SPR angle) to high accuracy.
By using a wedge of incident light and a fixed array of detectors, the SPR angle is monitored accurately in real time, with no physical movement of light source, sensor chip or detector.

The SPR signal is a direct measure of the angle of minimum reflected intensity. The unit of SPR response (resonance unit, RU), is an arbitrary unit, chosen so that 1 RU corresponds to a change in refractive index of $10^{-6}$, which in turn correlates with a shift in angle of about $10^{-4}$ degrees. For proteins on Sensor Chip CM5, 1 RU corresponds to a change in surface concentration of approximately 1 pg/mm². This correlation may however vary significantly for non-protein molecules and should be taken as a very approximate guideline.
Appendix A  The SPR detection principle
A.3  Biacore configuration
Appendix B  Theory of calibration-free concentration measurements

This appendix describes the theory behind calibration-free concentration measurements, that rely on fitting the observed binding rate during sample injection to a mathematical model of the interaction with analyte concentration as a fitted parameter. This approach delivers absolute concentration values without reference to a calibration curve.

B.1 Factors determining binding rates

B.1.1 Biochemical interaction rates

The rate at which an interaction proceeds is given by the difference between the forward (association) and reverse (dissociation) processes. For a 1:1 interaction

\[ A + B \xrightleftharpoons[k_d]{k_a} AB \]

where \( k_a \) and \( k_d \) are the rate constants for the association and dissociation respectively.

The association rate is given by \( k_a[A][B] \), and the dissociation rate is given by \( k_d[AB] \). The net rate of binding is

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

In Biacore, formation of complex is observed as an increase in response, measured in resonance units (RU). One interactant (the analyte, \( A \)) is supplied at a constant concentration during the sample injection. The available concentration of the second interactant (the ligand attached to the sensor surface, \( B \)) may be expressed in RU as the difference between the maximum analyte binding capacity \( R_{\text{max}} \) and the amount of complex formed \( R \).

Substituting these terms gives

\[ \frac{dR}{dt} = k_aC(R_{\text{max}} - R) - k_dR \]

where \( C \) is the concentration of analyte in the sample.

This represents the pseudo-first order kinetics observed for binding of analyte to surface-attached ligand with 1:1 stoichiometry. Similar model equations may be applied to more complex interaction models.
B.1.2 Mass transport processes

For analyte to bind to the sensor surface, the molecules must be transported from the bulk solution to the surface. This is a diffusion-controlled process. Under the conditions of laminar flow that apply in Biacore, the transport rate is directly proportional to the concentration of analyte in the bulk solution, with a proportionality constant called the mass transport coefficient \( k_m \) that varies with the cube root of the liquid flow rate.

Note that the transport rate is not influenced by the characteristics or amount of ligand immobilized on the surface.

B.1.3 What limits the observed binding?

In a given analysis situation, the observed rate of binding (i.e. the slope of the sensorgram) at any time will be determined by the relative magnitudes of the net biochemical interaction rate and the rate of mass transport. If interaction is much faster than transport, the observed binding will be limited entirely by the transport processes. Conversely, if transport is fast and interaction is slow, the observed binding will represent the interaction kinetics alone. When the rates of the two processes are of similar orders of magnitude, the binding will be determined by a combination of the two rate characteristics.

The net biochemical interaction rate (Section B.1.1) varies with the amount of available ligand sites on the surface, and is highest at the beginning of the injection. The mass transport rate, on the other hand, is constant throughout the injection since the analyte concentration in solution is constant. As a result, the relative importance of mass transport and biochemical interaction can change during the course of an injection: mass transport processes can be limiting at the beginning of the injection while interaction limits the observed binding rate at later stages (Figure B-1).

![Figure B-1](image.png)

**Figure B-1.** In a partially mass transport-limited situation, mass transport dominates at the beginning of the injection and interaction rate dominates late in the injection.
B.2 Technical aspects of calibration-free assays

Calibration-free assays rely on calculating the analyte concentration from the measured diffusion rate, using a known value for the diffusion coefficient of the analyte. In Biacore systems where this measurement approach is explicitly supported, this involves fitting observed binding data to a mass transport-limited 1:1 interaction model with a known value for the mass transport coefficient and an unknown variable for the analyte concentration.

The overall interaction process can be represented by the scheme

\[ A_{\text{bulk}} \xrightarrow{k_m} \frac{k_m}{k_m} A_{\text{surface}} + B \xrightarrow{k_d} AB \]

where \( k_m \) is the mass transport coefficient and \( k_a \) and \( k_d \) are the interaction rate constants. The rate of mass transport from bulk solution to the surface is given by

\[ \frac{d[A_{\text{surface}}]}{dt} = k_m[A_{\text{bulk}}] \]

In kinetic analyses, this relationship is used to determine \( k_m \) from the observed binding behavior at known values of \( [A_{\text{bulk}}] \). For calibration-free concentration measurements, a value is provided for \( k_m \) and the analysis calculates \( A_{\text{bulk}} \).

As a general recommendation, measurements should be made at two or more widely separated flow rates (for example 5 and 100 µl/min). The data is then fitted to a model with a global variable for analyte concentration (so that the model is constrained to find a single concentration value that best fits both curves simultaneously).

B.2.1 Mass transport in laminar flow systems

In the laminar flow conditions that apply in Biacore, the mass transport coefficient \( k_m \) is related to the analyte diffusion coefficient \( D \) by the expression

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

where
- \( D \) is the diffusion coefficient of the analyte in m²/s
- \( f \) is the volumetric flow rate of liquid through the flow cell in m³/s
- \( h, w, l \) are the flow cell dimensions (height, width, length in m)
Appendix B  Theory of calibration-free concentration measurements

B.2  Technical aspects of calibration-free assays

The mass transport coefficient $k_m$ has units of m/s. Adjusting for the molecular weight of the analyte and the conversion from measured RU to concentration units gives the Biacore-specific mass transfer constant $k_t$:

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

**Note:** The conversion constant $10^9$ is approximate and is only valid for protein analytes on Sensor Chip CM5.

### B.2.2 Diffusion coefficients for protein analytes

Values for the diffusion coefficient of many proteins may be found in the literature. The diffusion coefficient is determined by the size and shape of the molecule, so that values for a physically similar molecule may be used if the specific analyte is not listed (for example, the diffusion coefficient for all antibodies of IgG class will be practically identical since the molecules are essentially constant in size and shape).

Diffusion coefficients are directly proportional to the absolute temperature and inversely proportional to the relative viscosity ($\eta$) of the solution, so that if values can be found for one set of conditions, corresponding values can easily be calculated for the experimental conditions:

$$D = D_{ref} \times \frac{T}{T_{ref}} \times \frac{\eta_{ref}}{\eta}$$

where $D$ is the diffusion coefficient of the analyte

$T$ is the absolute temperature in Kelvin ($20°C = 293.15K$)

$\eta$ is the viscosity of the solvent

subscript $ref$ indicates reference conditions

Relative viscosity values should always be corrected for temperature if the experimental temperature differs from the reference value. The viscosity of common physiological buffer solutions (containing 0.15 M salt and no major additives such as glycerol) may however be considered equal to that of water, and correction for buffer composition is seldom necessary.

### B.2.3 Estimating diffusion coefficients from molecular properties

If there is no value available in the literature for the diffusion coefficient of the analyte being studied, a value may be estimated from the molecular weight and shape factor, or frictional ratio according to the equation below (this is a semi-empirical relationship based on Stokes law and the Einstein-Sutherland equation for molecular diffusion). The frictional ratio describes the extent of
deviation of the molecule from a sphere. A perfect sphere has a frictional ratio of 1.0. Globular proteins such as antibodies typically have values around 1.2. Moderately elongated proteins such as fibronectin and plasminogen typically have values in the range 1.6–1.9. For rigid elongated molecules like fibrinogen and tropomyosin, values are usually in the range 2–3.

\[
D = 342.3 \times \frac{1}{M^{1/3} \times f \times \eta_{rel}} \times 10^{-11}
\]

where

- \(D\) is the diffusion coefficient in m\(^2\)/s
- \(M\) is the molecular weight in daltons
- \(f\) is the frictional ratio
- \(\eta_{rel}\) is viscosity of the solvent relative to water at 20°C
  \((\eta_{rel} \text{ for water or buffer at } 25^\circ = 0.89)\)

Values for globular proteins with molecular weight around 100,000 daltons are typically of the order of \(6 \times 10^{-11} \text{ m}^2/\text{s} \) at 25°C.

**B.2.4 Reliability of calibration-free concentration measurements**

From the discussion above, it is evident that errors in the diffusion coefficient provided for evaluation of calibration-free concentration measurements will be transferred to corresponding errors in the measured concentration. Underestimation of the diffusion coefficient results in overestimation of the concentration by the error factor raised to the power of 2/3 (this follows from the relationship between \(k_m\) and \(D\), Section B.2.1). Thus for example underestimation of the diffusion coefficient by a factor of 2 will result in concentration values that are too high by a factor of about 1.6.

The reliability of measured diffusion coefficients reported in the literature must be assessed from case to case, on the basis of the validity of the experimental measurements.

Estimates of the diffusion coefficient from the molecular properties may be incorrect by a significant factor if the molecule is not globular and the frictional factor is unknown. Elongated and inflexible protein molecules may have frictional ratios as high as 10 or 20, so that some knowledge of the molecular shape is important for correct estimation.

Other factors such as viscosity and temperature have a relatively small effect on the diffusion coefficient. In general, errors in the value provided for the diffusion coefficient will often be less significant than other sources of experimental error such as less than perfect fitting of the binding data to the model or binding of mixed components to the sensor surface.
For evaluation of calibration-free concentration measurements, the mass transport coefficient $k_m$ is calculated from the diffusion coefficient, and then converted to the mass transport constant $k_t$ (Section B.2.1) which is used in fitting the experimental data to the diffusion-controlled interaction model. Uncertainties in the molecular weight and response-to-concentration conversion factor will affect the calculated concentration.

In terms of evaluating experimental data, consider the following aspects when assessing the reliability of the results:

- It is important that there is sufficient mass transport limitation in the sensorgram data. For binding that is completely limited by mass transport, the observed binding rate is proportional to the cube root of the flow rate (Section B.2.1). If the interaction properties limit the binding, the observed rate will be independent of the flow rate. Calibration-free concentration analysis involves measurements at two widely separated flow rates so that the influence of flow rate on binding rate can be assessed.

- In general, results will be most reliable when the initial binding rate at 5 µl/min is within the approximate range 0.3–15 RU/s and the ratio of initial binding rates at 100 and 5 µl/min is greater than 1.3.

- The experimental data should fit closely to the interaction model used to evaluate the concentration. If the fit is poor, the calculated concentration will be correspondingly unreliable.
Index

A
absolute response 10
accuracy 12, 79
acetate buffer 43
acetonitrile 53
acidic proteins 36, 39
activity of immobilized ligand 49
adjusting the assay range 62
advantages of Biacore 6
affinity 5, 25, 30, 62, 64
aldehyde coupling 36, 48
amine coupling 34, 45
amount of immobilized ligand 40, 63
amplifying the analyte response 30
analysis cycle times 30
analyte 9
unwanted binding 67
analyte binding capacity 30
antibodies
as capturing molecules 37
as detecting molecules 25, 71
as ligand 71
assay development 59
overview 7
assay documentation 78
assay formats 7, 19
assay performance 11, 59
assay requirements 59
assay settings 78
assay stability 74
assay versions 78
assessing regeneration 55
association rate 30, 87
audit trail 81

B
B_{50} 11, 16, 69
baseline after regeneration 56
baseline level 55
Biacore terminology 9
binding capacity 52
binding rate measurements 22, 61
biochemical interaction rate 88
biologically relevant concentration 5
biotinylation 41
biotinylation reagents 37
blank cycle 27
buffer composition 61
buffer exchange 44
bulk refractive index 21, 22, 30, 60
bulk response 54, 61

calibrated assays 19
calibration curve 75
calibration-free assays 19, 26, 62, 66, 89
analyte mixtures 71
capture 37
regeneration 53
capturing molecule 9, 37
carbohydrate 36, 48
carboxymethyl dextran 10, 33
carrier protein 26
chemical resistance 60
choice of ligand 29
choosing
 assay format 28
 immobilization method 41
 regeneration solution 53
cis-diols 48
coefficient of variation 13
colored samples 6
competing molecule 31
competition assays 19, 24
centration assay
advantages of Biacore 6
center of detecting molecule 65
centralion of ligand 40
contact time
for direct binding assays 63
for immobilization 46, 47, 48, 51
for inhibition assays 65
for regeneration 55
control samples 75
conversion between RU and
concentration 90
coupling buffer 43
cross-reactivity 11, 69
CV 13, 79
CV_{dose} 13
CV_{response} 13
cyanoborohydride 48
cystamine 46
cysteine 47

data storage 78, 81
degasser 60
detecting molecule 9, 20, 24, 28, 65
determining coupling conditions 44
  dextran 33
  diffusion coefficient 19, 89
diffusion properties 19, 26
diluting samples 60
direct binding assay 19, 20
  choice of ligand 29
  range 62
dissociation rate 87
dithioerythritol 46
dithiothreitol 46
documentation 78
DTE, DTT 46

E
  EDC 34, 45
electrostatic pre-concentration 44
ELISA 6
elongated molecules 91
end-point measurements 6
enhancement 19, 22, 64
enhancement reagent 30
epitope mapping 30
equilibrium dissociation constant 25
equipment qualification 77
ethanolamine 45
ethylene glycol 53
evanescent wave 83
evaporation 74

F
  file names 81
finding regeneration conditions 54
flow rate 27, 88
four parameter equation 16
frictional ratio 90
functional activity 28

G
  GCP see GxP
globular proteins 91
GLP see GxP
glycine-HCl buffer 53
glycoproteins 36
GMP see GxP
Good Clinical Practice see GxP
Good Laboratory Practice see GxP
Good Manufacturing Practice see GxP
GxP 5, 77

H
  haptoglobulin 26
  high affinity capture 37
  high molecular weight analytes 21
  hydrazine 36, 48

I
  IC50 11, 16, 25, 69
ICH guidelines 11, 77
immobilization 30
immobilization buffer 43
immobilization of ligand 34
immobilizing small molecules 38
immunological assays 23, 29
indirect assay 19, 24
inhibition 19
inhibition assays 19, 24, 31
  choice of detecting molecule 31
  choice of ligand 31
  optimizing 66
  range 64
interaction kinetics 88
interaction model 26
inter-assay precision 13
intermediate precision 12, 80
intra-assay precision 12
ionic strength 43, 50, 53
  for immobilization 39
isoelectric point 39

K
  kinetics 5, 87

L
  label-free measurement 6
laminar flow 88
ligand 9
  affinity for analyte 62
  concentration 43
  immobilization methods 34
  isoelectric point 39
ligand attachment 33, 43
ligand biotinylation 37
ligand capture 37
ligand immobilization
  acidic proteins 39
  aldehyde coupling 48
  amine coupling 45
  amount of ligand 40
  buffer pH 39
  choice of method 41
  conditions 38
  ionic strength 39
  ligand concentration 40
  procedure 45
  results 49
  surface activity 49
  surface thiol coupling 46, 47