Biacore™ application guide

Concentration measurements with Biacore™ systems

cytiva

Determination of biologically relevant concentrations is fundamental to many fields in both academic and industrial research, and is important as a tool for quality control in pharmaceutical development and production. The use of Biacore™ technology to measure concentration can provide advantages of speed, precision, automation, and simplicity in comparison with other techniques such as RIA and ELISA.

An essential feature of concentration assays that use Biacore™ systems (and of other interaction-based techniques) is that the measurements are based on binding of the analyte to the sensor surface or to a detecting molecule in solution, and the selectivity of the assay is determined primarily by the choice of interacting partner. Consequently, the technique measures the concentration of molecules that are able to interact, and the results may differ with different interacting molecules and from results obtained with approaches such as UV absorbance (A_{280}) or other total protein assays that do not take functional aspects in account.

The first method described in this Application Guide is calibrated concentration measurements, i.e., measurements that rely on a calibration curve obtained using standard samples (calibrants) with known concentrations. Calibrants may be commercial or in-house preparations, depending on the assay requirements. The second method described is calibration-free concentration analysis (CFCA) which does not require a calibrant or the generation of a calibration curve.

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Terminology and abbreviations

Calibrant

A substance used to construct a calibration curve by measuring the response obtained from solutions of known concentration.

Calibration curve

A plot of response against concentration for calibrant solutions, used to determine unknown concentrations.

Calibration-free concentration analysis (CFCA)

Calculates active concentration of proteins using their known diffusion coefficient and observed changes in binding rates at different flow rates under diffusion-limited conditions.

Control samples

Samples with known analyte concentration measured repeatedly at intervals throughout the assay, to monitor the stability of the assay. Calibrant solutions are frequently but not necessarily used as control samples[1].

Channel normalization samples

A solution of known concentration of analyte used to normalize responses between channels in multi-channel Biacore™ systems that support parallel analysis.

Reference sample

A sample with known and verified concentration (usually an official reference standard from an external supplier), used to establish the accuracy of the concentration assay. A reference sample is only required if the assay needs to be verified against an external standard.

Sample matrix

The sample matrix refers to components of the sample solution in addition to the analyte. A defined sample matrix such as buffer has a known composition. A complex matrix such as body fluids or cell cultivation medium is to some degree undefined.

DBA

Direct binding assay.

Detecting molecule

A molecule used in inhibition assays to reveal the amount of analyte in the sample. Commonly, the detecting molecule is larger than the analyte and gives a more reliably quantifiable response.

Enhancement

The use of a secondary interactant to enhance the specificity and/or sensitivity of a direct binding assay. Analogous to a sandwich assay in other techniques such as ELISA.

ISA

Inhibition in solution assay.

RIA

Radioimmunoassay

ELISA

Enzyme-linked immunosorbent assay

[1] The distinction between, for example, calibration samples and control samples is made in the way the assay method is constructed. See the documentation for your Biacore™ system for details.

First method

Concentration analysis using calibrant

Tips for calibrated concentration measurements

- Immobilize relatively high levels of ligand if possible (for example, 7000 to 12 000 RU for antibodies)
- If ligand may be attached by capturing, make sure that the same capturing level is obtained in each analysis cycle
- Include a reference flow cell in the method setup only if you want to use the results for additional purposes such as sensorgram illustrations. Reference subtraction is not relevant to concentration measurement.
- Use 2 to 8 calibrant concentrations that cover the required measurement range. Establish a suitable calibration range and number of calibrant concentrations as part of assay development.
- Prepare all samples, calibrants and control samples in the same sample matrix as far as possible. If this is not feasible, establish during assay development that differences between matrices do not interfere with the measurements.
- For parallel assays in multi-channel Biacore™ systems, use a concentration in the middle of the calibrated range for channel normalization. It is recommended to run a channel normalization cycle directly preceding each calibration cycle.
- If reference samples are used to verify the accuracy of the assay, run several dilutions to establish the accuracy over a range of concentrations
- Measure unknown samples at several dilutions to ensure that at least some samples fall within the concentration range of the assay. In the evaluation software for Biacore™ systems, results can be presented as the concentration in the undiluted sample, so that the results of different dilutions can easily be compared.

Assay formats for calibrated concentration measurements

Overview

Biacore™ systems support calibrated concentration measurements in four main formats:

- direct binding assay (DBA)
- DBA with enhancement
- inhibition in solution assay (ISA)
- surface competition assay

ISA and surface competition assays are both classed as inhibition assays.

Simple direct binding assays represent the most straightforward approach to measuring concentration with Biacore™ systems. In these assays, both with and without enhancement, the response is directly related to the analyte concentration.

The usefulness of direct binding level assays for measuring the concentration of small molecules may be limited due to the intrinsically low response obtained from small molecules. Inhibition assays represent an alternative approach in such cases. Two different formats are generally recognized, inhibition in solution and surface competition. In both cases, the response is inversely related to the concentration of analyte in the sample.

The formats are described in more detail in the following sections.

Direct binding assays (DBA)

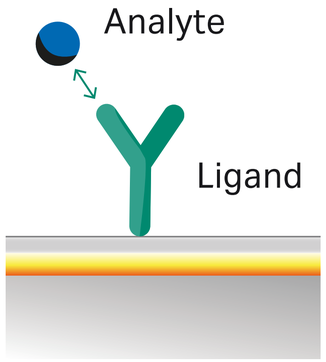
In direct binding assays, the ligand is attached to the sensor surface directly or by capture. The concentration of analyte in the injected sample is determined from either the sensorgram slope or the binding response after a fixed contact time. Sensorgram slope is usually measured early in the sample injection when the sensorgram is close to linear (see *Binding characteristics, on page 10*). Response is usually measured directly after the end of the sample injection to avoid bulk refractive index contribution from the sample matrix (see *Contact time and report point placing, on page 12*).

DBA with enhancement

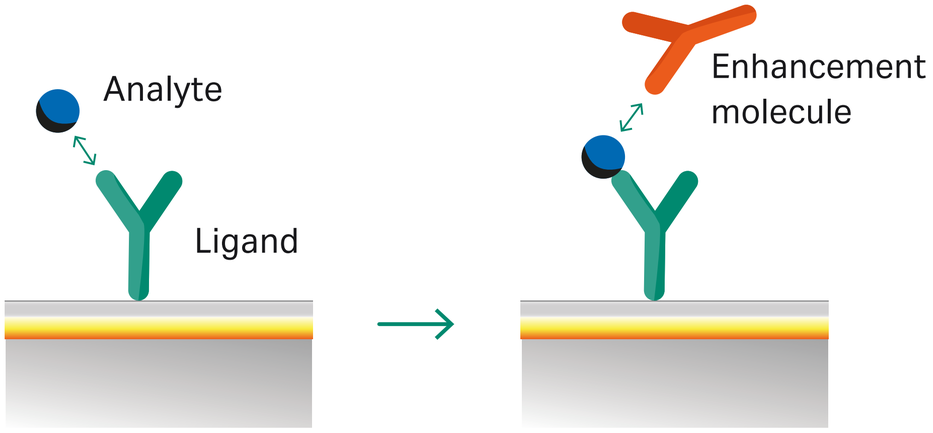
The sample injection in DBA can be followed by injection of an enhancement molecule, a secondary interactant that enhances the specificity and/or sensitivity of the assay. The role of enhancement in amplifying the analyte response has become less relevant as the sensitivity of Biacore™ systems has increased, and this aspect of enhancement is seldom relevant today. Enhancement of specificity however is a valuable feature of Biacore™-based assays, since the analyte is identified by specific binding to both ligand and enhancement molecule (for example, recombinant antibodies may be identified by both the recombinant tag and the antigen specificity).

A prerequisite for enhancement assays is that the analyte has distinct, noninterfering binding sites for the ligand and the secondary interactant. In practice, this restricts the usefulness of the approach to macromolecular analytes.

Assays using enhancement are analogous to sandwich assays that are part of the standard repertoire of immunological assay techniques in 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, which can provide valuable perspectives on the assay performance.



Direct binding assays (DBA)

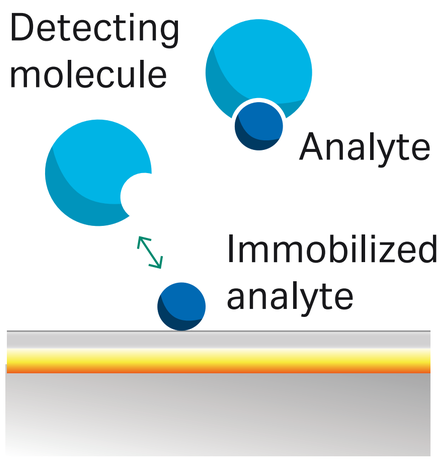


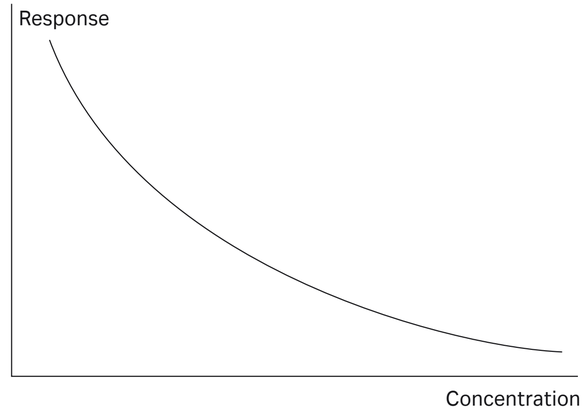
DBA with enhancement

Inhibition in solution assays (ISA)

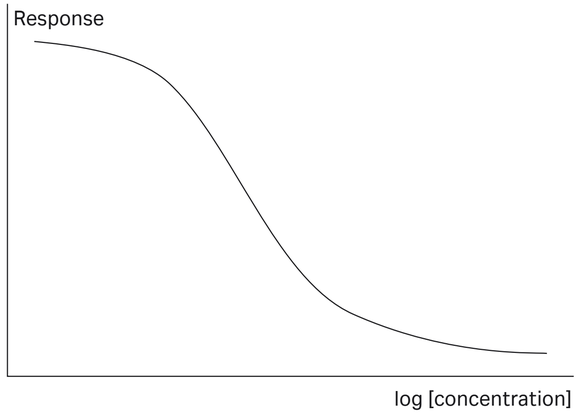
Inhibition in solution assays (ISA) exploit the ability of the analyte to inhibit the binding of detecting molecule to the surface, and are typically used for low molecular weight analytes. The analyte or an analogue thereof is attached to the surface as the ligand, while the detecting molecule is a macromolecule (for example an antibody) that binds specifically to the analyte. The detecting molecule is mixed at a constant known concentration with the samples, with the detecting molecule in excess of the maximum analyte content. Samples that contain no analyte will permit maximum binding of the detecting molecule to the surface. Any analyte in the sample will bind to the detecting molecule in solution and inhibit binding to the surface. The observed response, which is derived from detecting molecule binding to the surface, is inversely related to the amount of analyte in the sample.

Ideally, the detecting molecule should be monovalent, so that detecting molecules with bound analyte in solution cannot bind to the surface-attached ligand. In practice, however, monoclonal antibodies are commonly used as detecting molecules, in spite of their bivalent binding properties, and the assay format still works reliably.





The response in inhibition assays is inversely related to the concentration.



Calibration curves are often shown with a logarithmic concentration axis, to visually expand the low concentration region.

Surface competition assays

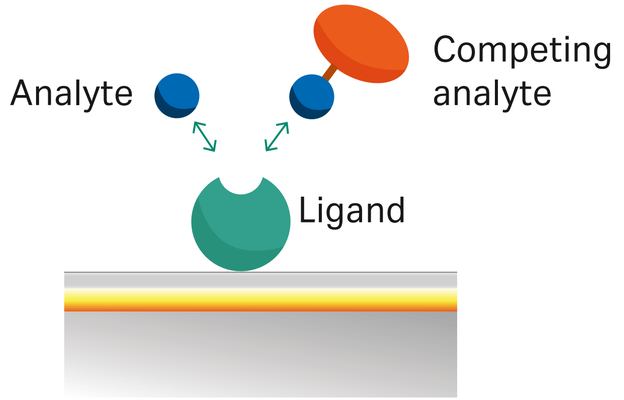
In the surface competition approach, a binding partner to the analyte is used as ligand, and a high molecular weight analogue to the analyte (typically analyte conjugated to a carrier protein) is added in constant amount to the samples to be measured. The analyte competes with the high molecular weight analogue for binding to the ligand. The measured response is the sum of the contributions from analyte and high molecular weight molecule: if the analyte is small relative to the analogue, the response is dominated by the contribution from the analogue. The observed response is inversely related to the amount of analyte in the sample.

The surface competition approach can have advantages over the more common ISA format in situations where immobilization of the analyte on the sensor chip surface presents problems. Low molecular weight analytes that are typically addressed with inhibition assays are not always amenable to the immobilization, but can more easily be conjugated to a carrier protein.

Requirements for the carrier protein and conjugated analyte are as follows:

- The carrier protein is large enough to give a response clearly distinguishable from that given by analyte alone
- The carrier protein does not bind on its own to the sensor chip surface
- Conjugation of the analyte does not interfere with binding to the ligand
- The level of conjugation is kept low (typically about 1 analyte per carrier protein molecule) to minimize avidity effects

Cheap and readily available proteins such as transferrin or haptoglobulin are frequently suitable as carrier proteins. Do not use serum albumin since it binds to many small molecules.



General considerations

Binding characteristics

Interaction rates monitored in Biacore™ systems may be limited by kinetic properties of the interaction or by the rate of mass transport of analyte to the sensor surface, or by a mixture of the two (see the Application guide *Principles of kinetics and affinity analysis* for more details). Interaction-limited binding rates are determined by the properties of analyte and ligand as well as the concentration of analyte. Mass transport rates, on the other hand, are determined by the diffusion properties of the analyte (i.e., physical size and shape) and are independent of interaction properties.

It is generally preferable to measure concentration under mass transport-limited conditions if possible, so that the kinetic interaction properties do not affect the calibration curve or sample measurements. This can be particularly relevant, for example, in measurements involving antibodies: different antibody clones may have widely different interaction characteristics with the antigen, but all antibodies in a given subclass have essentially the same size and shape.

In addition to providing independence from kinetic properties, measurement under mass transport-limiting conditions helps to establish robust assays, since the sensorgram is linear as long as mass transport is the dominant limiting factor.

Use the following conditions to favor mass transport limitations:

- Relatively high ligand levels (typically around 10 000 RU for proteins with molecular weight 150 000)
- Relatively low flow rates for the sample injection (recommended 10 µL/min)

Sensor surface preparation

Immobilize relatively high ligand levels on the sensor surface if possible (typically 7000 to 10 000 RU for protein ligands with molecular weight 30 000 to 150 000). This will help to give confidently measurable responses from low analyte concentrations, and will favor mass transport-limited binding (see *Binding characteristics, above*). Lower immobilization levels may be necessary in assays that use ligand capture or enhancement formats, to avoid crowding effects and steric hindrance on the sensor surface.

Reference surface

A reference surface is not normally used in concentration measurements. Differences in non-specific binding of sample matrix components to the active and reference surfaces can introduce errors in the measurements, and may be difficult to control with complex sample matrices.

Sample preparation

The SPR technology in Biacore™ systems allows measurements to be made on colored, turbid, or even opaque samples. Particulate samples can also be used provided that particles and aggregates are not larger than 20 µm diameter and do not tend to settle out of suspension.

In general, samples should be in aqueous medium, with the addition of low concentrations of organic solvents to maintain solubility if necessary. Information concerning chemical resistance of the flow system is provided in the documentation for the system. Samples, control samples and calibrants should ideally all be prepared in and diluted where necessary with running buffer. Samples may need to be diluted so that the concentration falls within the measuring range of the assay.

Samples in complex matrices such as body fluids or cell cultivation media may be partially purified using standard fractionation procedures if desired. However, one of the advantages of using Biacore™ systems for concentration measurement is that complex samples can be analyzed directly. Where possible, calibrants and control samples should be prepared in the same complex matrix as the samples (for example, cell cultivation media).

When the analyte is presented in a complex medium, enhancement techniques can be valuable for confirming specificity of the response (see *DBA with enhancement, on page 7*).

More or less extensive assay development work may be required to establish the validity of assay for samples in complex matrices. This can include tests for:

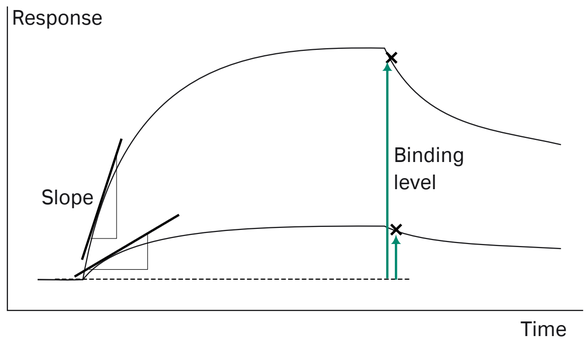
- Non-specific binding of matrix components to the sensor surface, tested by performing the assay with analyte-free matrices
- Recovery of analyte from matrix samples spiked with known analyte concentrations (see *Accuracy and recovery, on page 22*)
- Tests of the assay linearity in varying matrix concentrations (see *Linearity, on page 23*)

Contact time and report point placing

Response values used for concentration measurement may be either binding level or sensorgram slope at a specified report point. The choice is made during evaluation, and is applied automatically to calibrants, control samples, and unknown samples. Contact time for sample injections is set according to the report point placing that will be used. Generally recommended values are 60 to 180 s.

Binding level measurements should preferably be obtained from a report point shortly (5 to 30 s) after the end of the sample injection, to avoid bulk refractive index contributions. If analyte dissociation is rapid, it may be necessary to place the report point before the end of the injection. In such cases, careful matching of the bulk refractive index in samples and running buffer to minimize bulk contributions becomes more important.

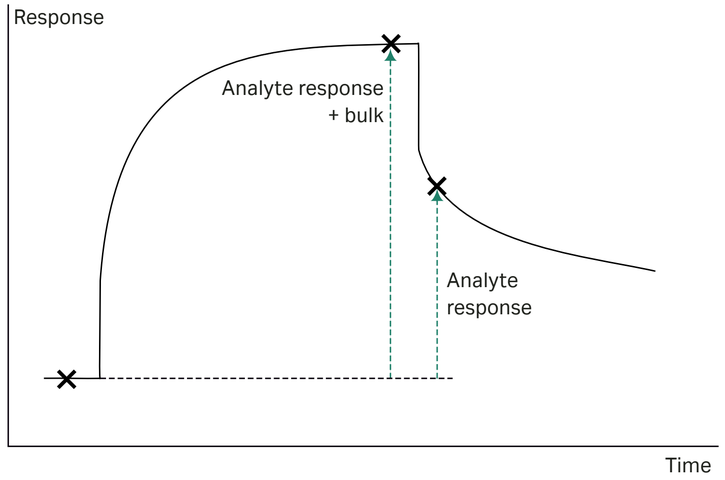
Slope measurements are best obtained from report points early in the sample injection, when mass transport is most likely to be limiting (see *Binding characteristics, on page 10*) and the sensorgram is close to linear. Slope measurements are generally most useful for macromolecular analytes that give confidently measurable responses early in the sample injection. Slope measurements are not affected by bulk refractive index contributions.



Concentration measurements may be based on sensorgram slope or binding levels.

Regeneration

Efficient regeneration of the surface between cycles is a requirement for reliable concentration determinations. Analytes that do not dissociate rapidly enough must be removed by injection of regeneration solution. 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. For more information refer to *Biacore™ Sensor Surface Handbook*.



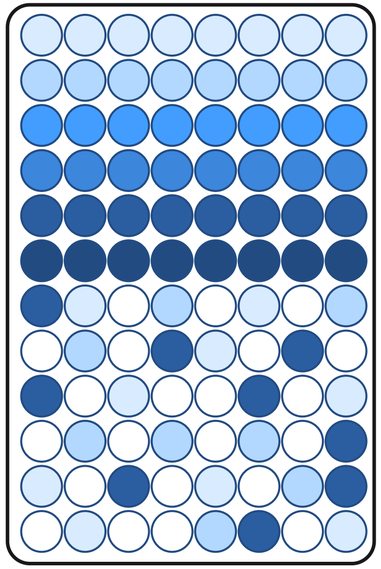
Report points for binding levels should preferably be placed after the end of the sample injection, to avoid bulk contributions.

Experimental setup

Serial assay formats

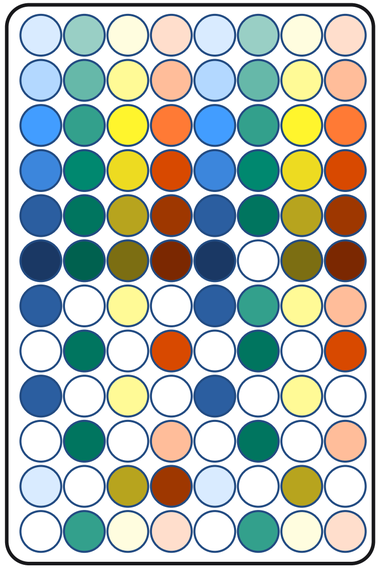
In Biacore™ systems with a single injection needle, assays are necessarily performed in **serial format** with one measurement in each cycle. Serial format in systems with multiple channels can be used either to increase the throughput for measurements of a single analyte or to increase assay diversity by measuring multiple analyte-ligand pairs in a single run. Regardless of how the assay is set up, evaluation is performed within channels.

Single analyte (throughput)



Each channel provides a calibration curve and sample measurement for the same analyte. Evaluation is performed within channels.

Multiple analyte (diversity)



Each channel provides a calibration curve for a different specific analyte.

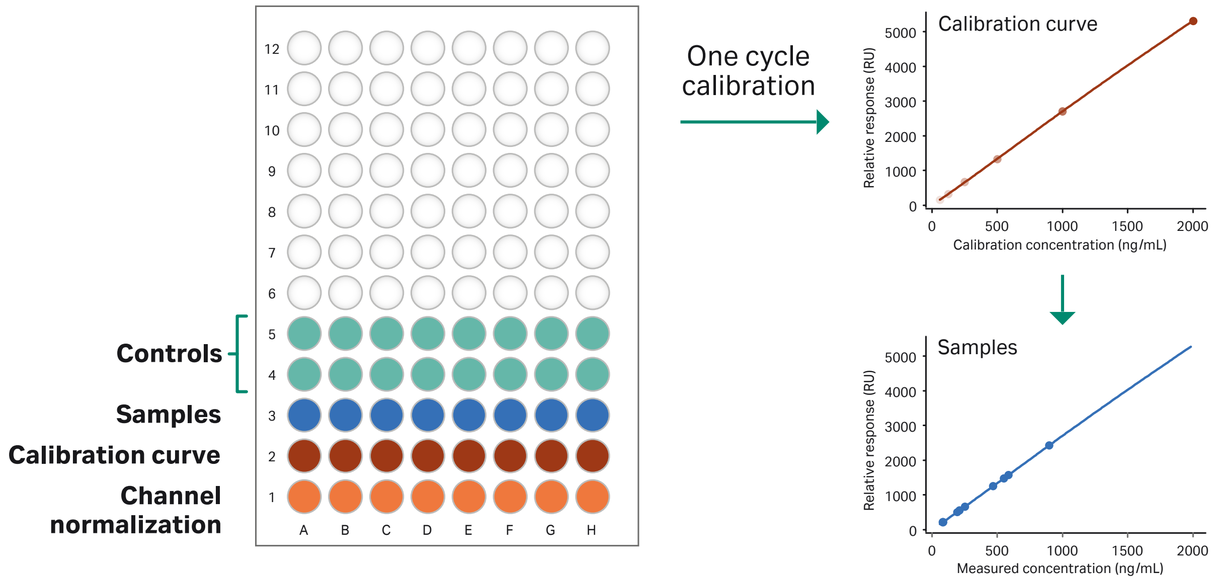
Parallel assay format

Systems with multiple injection needles can also be used for DBA in **parallel format**, where different concentrations of the same calibrant are run in different channels, and the calibration curve is constructed from the different channels in a single cycle. Parallel assays should include a channel normalization cycle directly preceding each calibration cycle (see *Channel normalization, on page 15*).

Calibration curves

Calibration curves should be run at the beginning of the assay, and may be repeated at intervals to adjust for drift in the calibration performance (for example, progressive loss of ligand activity). The frequency of repeated calibration depends on the stability of assay performance and the number of samples to be measured, and should be determined during assay development.

Calibrants may be run in increasing, decreasing or random concentration order, and the number of calibration points may vary between different assays. The range of calibrant concentrations is established during assay development, and should cover the expected range of sample concentrations (after any dilution that may be necessary) according to the requirements of the assay. In general, calibration curves cannot be extrapolated, and samples that fall outside the calibration range can only be reported as above or below the highest and lowest callibration point respectively.



Parallel assay format

Channel normalization

Channel normalization compensates for variations between channels in parallel assays, where calibrants are run in different channels in the same cycle. The channel normalization cycle injects the same sample concentration in each channel, and compensation is performed so that the normalized responses are the same from the same sample concentration. Channel normalization is only used in parallel assays.

A channel normalization cycle should be included directly preceding each calibration cycle, to allow compensation for response variations between channels. Compensation is performed automatically in evaluation software where the parallel format is supported.

Channel normalization is not recommended for assays that use ligand capture since the capture levels can vary between cycles. For parallel assays using capture it is important that capture levels are consistent between channels and cycles.

Control samples

Control samples may be run at intervals to monitor the stability of the assay. Control samples may be internal standards with known analyte concentrations used to verify consistency of the results between separate assay runs, or external reference samples used to verify the accuracy of the assay (see *Accuracy and recovery, on page 22*).

Workflow

A suggested workflow for concentration measurement with Biacore™ systems is described on the right.

Step	Action
1.	Dock and prepare an appropriate sensor chip using standard methods.
2.	Run 1 to 10 startup cycles with regeneration if applicable. Use a sample that mimics an analyte concentration in the high region of the calibration curve (a relatively high analyte concentration for DBA and a low or zero analyte concentration for ISA).
3.	For parallel DBA assays in multi-channel systems, run a channel normalization cycle directly after the startup cycles, directly before the calibration cycle. Use a sample of calibrant or known analyte, diluted in running buffer to give a response in the middle of the measuring range of the assay. Use the same sample in all channels.
4.	Run a calibration curve with 2 to 8 calibrant solutions. Repeat the calibration curve at intervals throughout the assay to allow for drift in the assay performance. In parallel DBA assays, repeat the channel normalization cycle directly before each calibration cycle.
5.	Run control samples according to assay requirements. Control samples are run at the beginning and end of the assay and repeated at intervals to monitor the assay stability.
6.	Run reference samples if applicable. Reference samples are used to evaluate the accuracy of the assay (see *Accuracy and recovery, on page 22*).
7.	Run the unknown samples, diluted if necessary so that the concentration of at least one dilution falls within the measuring range.

Parameters and settings

The table below summarizes recommended settings for DBA.

Parameter	Recommendation	Comments
Flow rate for capture	10 µL/min	Minimize ligand consumption
Contact time for capture	60 to 180 s	According to assay requirements
Flow rate for sample	10 µL/min	Minimize sample consumption
Contact time for sample	60 to 180 s	Dependent on binding rate. Longer contact times can increase assay sensitivity.
Dissociation time for sample	0	Dissociation time is not relevant for concentration measurement
Flow rate for enhancement	10 µL/min	Minimize reagent consumption
Contact time for enhancement	120 s	Adjust according to the rate of enhancement reagent binding
Flow rate for regeneration	30 µL/min	Use the same flow rate as the sample injection if the response stabilizes slowly after regeneration
Contact time for regeneration	30 to 180 s	Depending on assay requirements

Adjusting the operating range

Introduction

The operating range of an assay is defined in terms of lower and upper limits that can be measured with acceptable performance (see *Performance criteria, on page 21*). The limits are determined as described in the table to the right.

Assay format

DBA	ISA	Determining factors
Lower limit	Upper limit	Lowest reliably measurable response
Upper limit	Lower limit	Affinity and concentration of ligand/detecting molecule Injection time for sample

The ways in which the operating range can be adjusted differ according to the assay format.

Direct binding assays

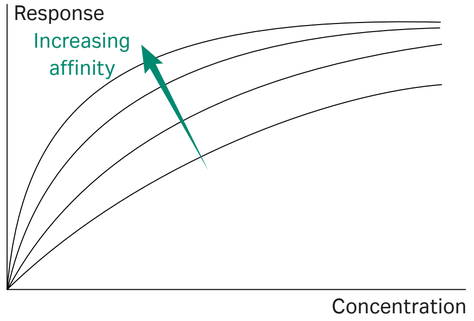
To optimize the range of a direct binding assay with or without enhancement, determine a calibration curve to establish the approximate range of the assay and then use the principles described below to make appropriate adjustments. If the range requirements cannot be met by adjusting the contact time or ligand level, you may need to review your choice of ligand and/or assay format.

For best performance over the whole range, the operating range of DBA should be restricted to the linear region of the calibration curve. In practice, some curvature of the calibration curve at high concentrations may be acceptable.

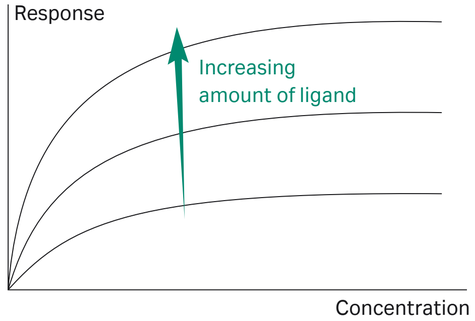
The lower limit for DBA with or without enhancement is determined by lowest reliably measurable response, which is a property of the instrument and cannot usually be adjusted. While enhancement can theoretically be used to amplify the analyte response, the approach requires separate and independent binding sites for the ligand and the enhancement molecule, and cannot normally be used with small molecules where the need for amplification is greatest. If the lower limit of the operating range for DBA is unacceptable, consider using a different format.

Two factors may be exploited to adjust the upper limit operating range of DBA:

- Increasing the amount of immobilized ligand increases the response obtained for a given analyte concentration, allowing measurement over a wider range with maintained assay precision
- Using a ligand with a higher affinity gives a higher response for a given analyte concentration within the linear range of the calibration curve, but results in a calibration curve that flattens out at lower concentrations. As a result, the operating range is shifted towards lower analyte concentrations.



Using a ligand with higher affinity moves the calibration curve towards lower analyte concentrations.



Using higher levels of immobilized ligand increases the analyte response but has little effect on the shape of the calibration curve.

DBA with enhancement

For assays using enhancement, 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 so that binding is complete with short contact times.

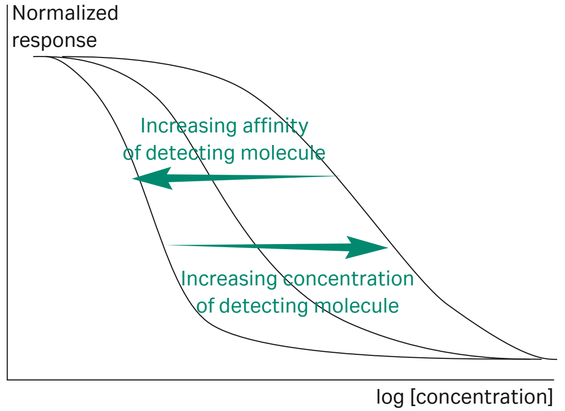
Inhibition assays

Since inhibition assays measure the amount of free detecting molecule in solution, the calibration curve is inverted in comparison with DBA, with high responses at low analyte concentrations.

The useful range of an inhibition assay is determined by the affinity of the detecting molecule for the analyte (both free in solution and attached to the surface) and the concentration of detecting molecule. The affinity for analyte in solution is assumed to be similar to that on the surface.

- Higher concentrations of detecting molecule give a higher response at a given analyte concentration, but compress the calibration curve at high concentrations. Reducing the detecting molecule concentration can extend the operating range towards lower analyte concentrations, provided that the response can still be confidently measured.
- Higher affinity of the detecting molecule for the analyte allows measurement at lower analyte concentrations but also results in a narrower operating range

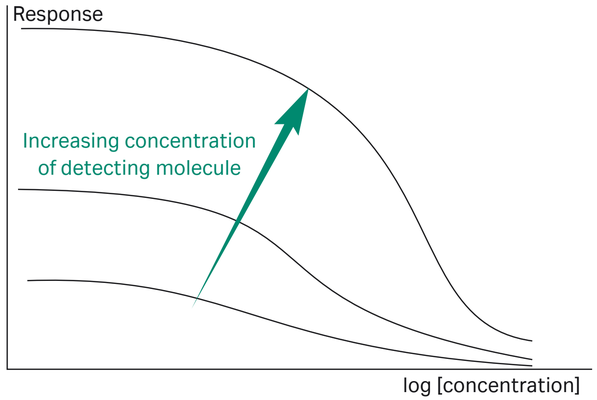
Follow the steps to the right for a recommended workflow for optimizing an inhibition assay.



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 analyte concentrations.

Responses are normalized for comparison.



Increasing the concentration of detecting molecule increases the maximum response which may allow measurement at lower analyte concentrations.

Step	Action
1.	Prepare the sensor chip by immobilizing the analyte or analogue.
2.	Inject the detecting molecule at a range of concentrations with no added analyte. Select a concentration that gives a suitable response level (typically 100 to 2000 RU) with a reasonably short contact time.
3.	Run a series of samples containing analyte at known concentrations with the selected concentration of detecting molecule, covering the required operating range of the assay.
4.	If the highest analyte concentration does not inhibit binding of detecting molecule sufficiently (ideally, reduce the response level to baseline), try using a lower concentration of detecting molecule. You may be able to extend the contact time to compensate for the lower response levels. It is not always possible to achieve complete inhibition.
5.	Examine a plot of response against analyte concentration to establish that the high and low ends of the operating range provide sufficient resolution in terms of analyte concentration.
6.	If the required assay performance cannot be met by adjusting the concentration and contact time of detecting molecule, review the choice of detecting molecule with respect to affinity for the analyte.

Evaluation

Evaluation of concentration measurements is performed using predefined functions or evaluation methods in the evaluation software. Details differ between different Biacore™ systems and are described in the documentation for the respective system. All procedures and methods however create a table with the results for calibrants, control samples, and samples. The procedure is based on simply reading the concentration corresponding to the sample response from the calibration curve, and is the same for DBA and ISA. Statistical parameters and replicate averages are included in the result table.

User intervention in the evaluation procedure involves general quality control, parameter selection and management of calibration trends, as described below.

General quality control

Before applying the evaluation procedure, check the results for general quality. Depending on the evaluation software used, quality control actions such as exclusion of outliers may or may not affect existing evaluation results. See the system documentation for details.

- Examine the sensorgrams for disturbances that affect either the baseline or the report point used for evaluation. Exclude any disturbed cycles. If the evaluation report point is disturbed in multiple cycles, consider creating another report point in an undisturbed region.
- Examine the baseline plot for inadequate regeneration, loss of immobilized ligand or similar trends. Exclude any outliers.
- Examine the sensorgrams for binding to the reference surface if one is included in the assay
- If channel normalization is used for parallel assays, examine the channel normalization cycle(s). Ideally, response variation between channels should be small in relation to the measured responses. Repeated channel normalization cycles should show a similar pattern if variation between channels. Exclude any obvious outliers.

Evaluation settings and parameters

Once the evaluation procedure has been applied, the following settings and parameters to the right can be adjusted as required for the particular assay.

Channel normalization for parallel assays will be applied automatically if channel normalization cycles are included in the assay. Exclude normalization cycles from the evaluation session if you do not want to use channel normalization.

Calibration trends

Calibration trends is a tool that analyzes trends in repeated calibration curves by fitting trend lines to the measured calibration points. Control samples and unknown samples are evaluated using virtual calibration curves interpolated for each cycle between the measured curves.

Use calibration trends if there is a clear progressive change in the calibration curves during the course of the assay, for example, as a result of loss of surface activity.

Calibration trends are only supported in some Biacore™ systems.

Setting	Comments
Report point	Choose the report point to use for response measurement. The default setting is usually just after the end of the sample or enhancement injection.
Response type	Choose between response level and sensorgram slope (see *Contact time and report point placing, on page 12*).
Calibration curve	If repeated calibration curves have been run, choose whether to use preceding or average curves. Software for some Biacore™ systems can also use calibration trends for evaluation (see *Calibration trends, to the left*).
Fitting function	Choose between a linear fit and a 4-parameter fitting function for fitting calibration curves to experimental points.

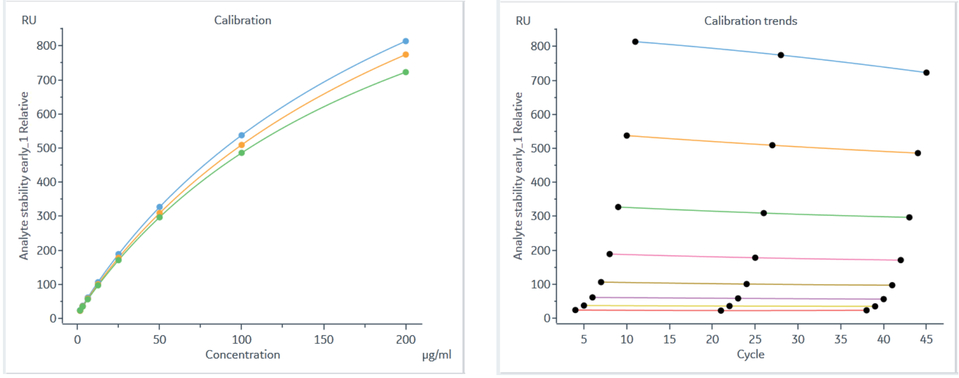


Illustration of calibration trends taken from Biacore™ Insight Evaluation Software. The left-hand panel shows 3 calibration curves measured at different times during the assay. Calibration trends in the right-hand panel show the trends over cycle number for each callibrant concentration.

Performance criteria

Concentration assays should be designed so that the performance is in accordance with the purpose of the measurements. This section gives brief descriptions of essential performance criteria based on the ICH recommendations[1]. More details may be found in the ICH document.

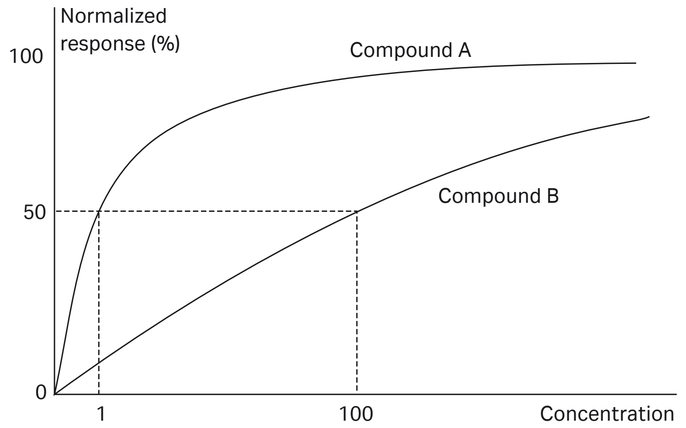
Specificity, selectivity and cross-reactivity

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.

Selectivity refers to the ability to measure a class of analyte species without distinguishing the individual members of the class. Selectivity has therefore a wider meaning than specificity, although the terms are sometimes used interchangeably.

Cross-reactivity is a quantitative measure of specificity and selectivity, and is expressed formally for Biacore™-based assays in terms of the ratio of the concentrations of different analytes that give 50% binding saturation (B_{50}). A compound that requires 100 times higher concentration than the analyte to reach the B_{50} value is said to show a cross-reactivity of 1%.

[1] International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. *Q2(R1) Validation of analytical procedures: text and methodology* (2005)



B_{50} values are 100 for compound B and 1 for compound A. Compound B shows 1% cross-reactivity with compound A.

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 reference samples in comparison either with the results of an independent reference assay or with quoted values for the reference sample.

A relevant consideration in this respect is the method used to determine the quoted concentration of the reference sample. Biacore™ systems only measure analyte that is capable of binding to the ligand or detecting molecule: this may differ from values based on, for example, determination of protein content. Discrepancies between results from Biacore™ systems and reference values are strictly inaccuracies in the assay, but may in some circumstances be desirable.

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.

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 instruments etc. Ruggedness is an alternative term for intermediate precision.
- **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.

Note: Accuracy and precision are frequently confused, although they are clearly distinguished in their formal definitions. An assay that is precise but not accurate will always give the same incorrect answer, while one that is accurate but not precise will give an approximate but correct answer.

Assay precision 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

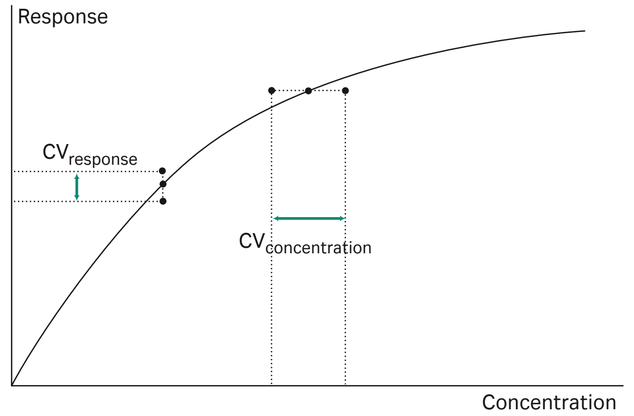
$$SD = \sqrt{\frac{1}{(n-1)}\sum (y_i - \bar{y})^2}$$

Parameter	Meaning
n	Number of measurements
y_i	Response for a given measurement
y	Average response for the measurement series

The coefficient of variation is given by

$$CV(\%) = \frac{SD}{mean} \times 100$$

CV values and other measures of variation may be related to either concentration or response values. $CV_{response}$ reflects the consistency of response values for a given concentration, while $CV_{concentration}$ reflects the confidence with which a given response value can be related to analyte concentration. Values for CV vary over the dynamic range of the assay unless the calibration curve is linear. In general, $CV_{concentration}$ values are lowest in the middle of the range, and increase towards the bottom as accurate measurements become more difficult, and towards the top as the calibration curve flattens out at the top. $CV_{response}$ values, on the other hand, are frequently low even at the top of the range. $CV_{concentration}$ is in general regarded as a better criterion of assay performance.



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, a linear relationship cannot be obtained even after mathematical transformation. 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™ systems uses either linear or 4-parameter functions for creating a calibration curve from the measurement points.

In quantitative terms, linearity is expressed as the regression coefficient for fitting the data points to a straight line. An alternative approach when a linear function is not available is to determine the regression coefficient for a plot of the measured calibrant concentrations against the expected (known) values. This plot should always be a straight line regardless of the shape of the calibration curve.

Limit of detection (LOD)

The **limit of detection (LOD)** is the lowest analyte concentration that can be detected but not necessarily determined with acceptable performance. 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 the mean blank value plus 3 × SD, where SD is the standard deviation of replicate measurements on blank samples.

Note: The LOD determined from measurements in the absence of analyte does not account for any variation in source, composition or preparation of samples. These parameters affect the limit of quantitation, described on the following page.

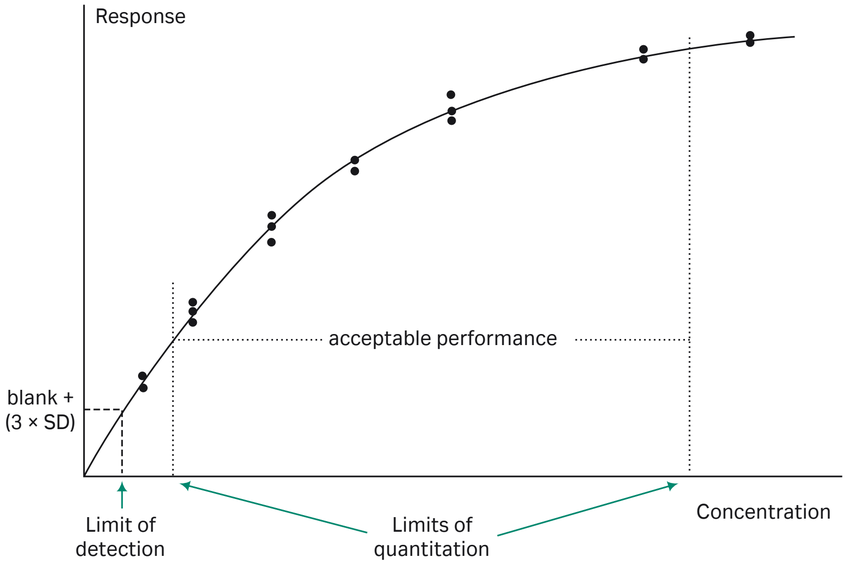


Illustration of limit of detection and limit of quantitation.

Limits of quantitation (LOQ)

The upper and lower **limits of quantitation** (ULOQ and LLOQ) are the highest and lowest analyte concentrations that can be measured with acceptable linearity, precision and accuracy. Acceptable limits are set according to the purpose and requirements of the assay. Assay procedures which permit measurement on diluted samples may not have an explicit ULOQ, but the limits for the assay itself are relevant in defining the extent of dilution that is permitted.

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.

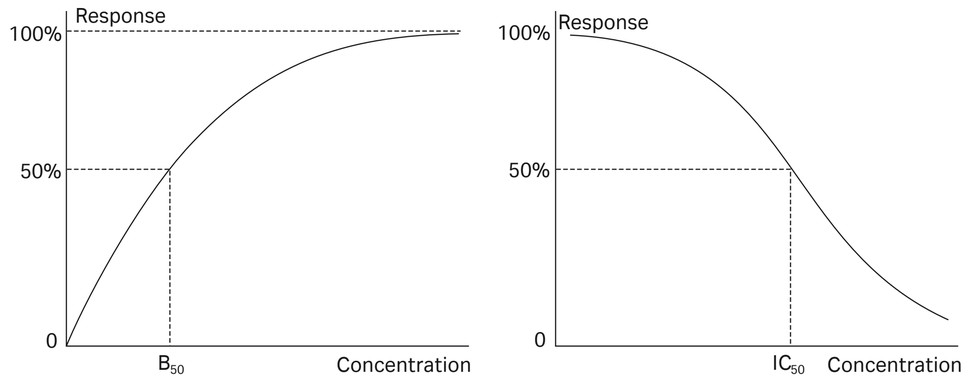
If the demands on documented assay performance are less stringent, an initial estimate of the LLOQ may be determined as the mean blank value plus 10 × SD, where SD is the standard deviation of replicate measurements on blank samples. This value can then be verified by using a relatively small number of measurements on samples containing analyte.

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.

B_{50} and IC_{50}

A parameter that is often quoted for an assay is the analyte concentration that gives 50% of the maximum response (B_{50} for a DBA, IC_{50} for ISA). This value is strictly not a performance criterion, but indicates the order of magnitude of analyte concentrations that the assay can handle. Note that the B_{50} and IC_{50} values do not necessarily represent the midpoint of the concentration range.



Robustness

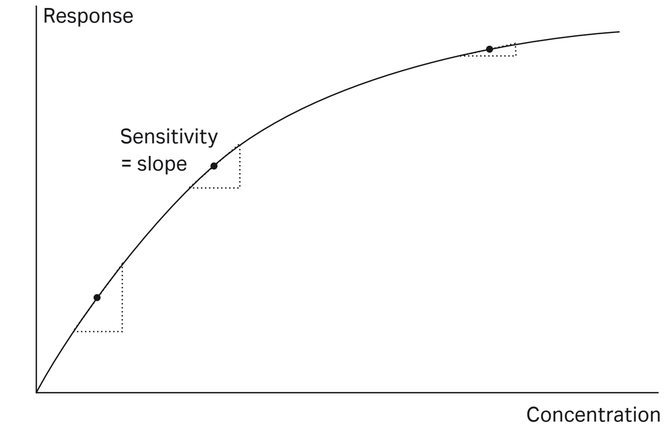
The **robustness** of an assay is a measure of its capacity to remain unaffected by variations in method parameters. Robustness is related to intermediate precision. While intermediate precision refers to the effect of unintentional variations between assay occasions, operators and so on, robustness 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.

Sensitivity

The **sensitivity** of an assay is not included among recommended performance criteria for assay validation, but is defined here because the term is often used in several different and to some extent conflicting senses.

Formally, sensitivity is defined as the slope of the calibration curve, expressed for Biacore™-based assays in response units per concentration unit. 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 LLOQ (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.



Second method

Calibration-free concentration analysis

Tips for calibration-free concentration analysis

- Immobilize relatively high levels of ligand if possible (for example, 7000 to 12 000 RU for antibodies)
- To ensure that binding is mass transport limited, run more than one dilution of the samples in the same experiment
- Assay set up is as simple as diluting samples to 5–10 nM in assay buffer followed by injection at two different flow rates, typically at 5 and 100 µL/min.
- No need to remove buffer components such as urea and glycerol
- The diffusion coefficient can be calculated using the Diffusion Coefficient Calculator available at cytiva.com/biacoretools.

Setting up calibration-free concentration assays

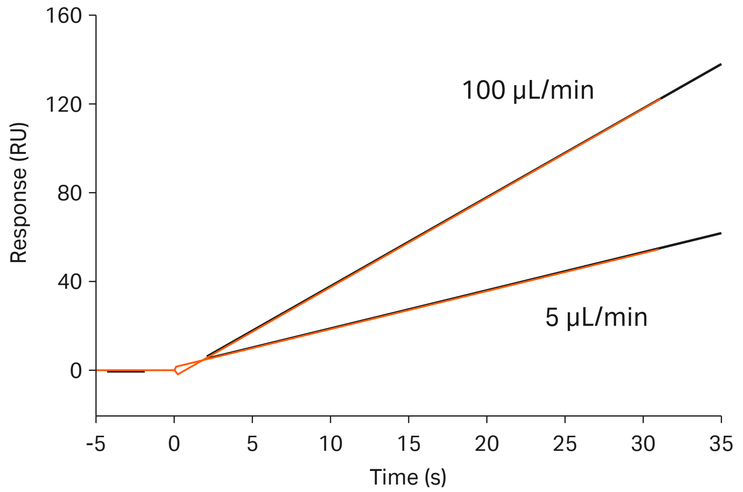
Some Biacore™ systems directly support calibration-free concentration measurements. In principle, you could apply calibration-free concentration measurements in any system that can measure binding in partially mass transport-limited conditions.

We recommend an approach that involves analyzing the interaction with the same sample during short sample injections (recommended 36 s) using at least two different flow rates. Normally, it's recommended to use flow rates as widely separated as the system will allow (e.g., 5 and 100 µL/min). We recommend a blank cycle (injecting buffer instead of sample) for each flow rate. Subtracting this blank from the sample cycles helps to eliminate systematic variations in the response, and improves the robustness of the assay.

You should always perform calibration-free measurements on a sensor chip that will let enough ligand immobilize. Sensor Chip CM5 supports this, and you can also use some pre-immobilized sensor chips such as Sensor Chip Protein A. If you use a pre-immobilized sensor chip, then you should perform analysis without using reference subtraction. Evaluation also depends on a default conversion constant between RU and surface concentration (see *Theory of calibration-free concentration measurements, on page 29*), which is valid for Sensor Chip CM5. You might need to adjust the conversion constant for other sensor chips.

To favor mass transport-limited interaction, the levels of immobilized ligand should be high (typically 5000–10 000 RU for proteins). For best results, the initial binding rate at 5 µL/min should be between 0.3 and 15 RU/s (corresponding to concentrations for typical proteins in the range of 0.05–5 µg/mL). The ratio of binding rates at 100 and 5 µL/min should be higher than about 1.3. In practice, the assay setup is often as simple as diluting the samples to 5–10 nM in assay buffer before injecting them at two different flow rates (5 and 100 µL/min) over the immobilized target.

The sensorgrams at both flow rates should be approximately linear during the first 30 seconds of interaction – if they're not linear, your evaluation will be less robust. When choosing a ligand for calibration-free assays, we recommend the same considerations as for direct binding assays using a calibration curve. We detail how to evaluate calibration-free concentration assays in the section *Theory of calibration-free concentration measurements, on page 29*. The figure shows an example of a calibration-free concentration analysis with linear curves at both 5 and 100 µL/min.



Example of a calibration-free concentration analysis with linear curves at both 5 and 100 µL/min.

Theory of calibration-free concentration measurements

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

Factors determining binding rates

Biochemical interaction rates

You can determine the rate of an interaction by calculating the difference between the forward (association) and reverse (dissociation) processes.

For a 1:1 interaction where k_a and k_d are the rate constants for the association and dissociation respectively:

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

The association rate is $k_a[A][B]$, and the dissociation rate is $k_d[AB]$. The net rate of binding is:

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

In Biacore™ systems, complex formation is observed as an increase in response and is measured in resonance units (RU). Interactant A (the analyte) is supplied at a constant concentration during the sample injection. You can calculate the available concentration of the second interactant (the ligand attached to the sensor surface, B) by subtracting the amount of complex formed R from the maximum analyte binding capacity R_{max}. The available concentration is expressed in RU.

Substituting these terms gives the following equation, where C is the concentration of analyte in the sample:

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

This represents the pseudo-first order kinetics observed for analyte binding to surface-attached ligand with 1:1 stoichiometry. You can apply similar model equations to more complex interaction models.

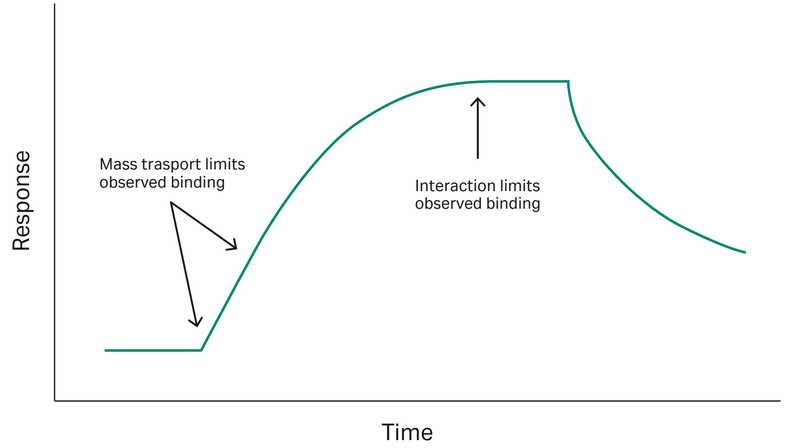
Mass transport processes

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. In the laminar flow conditions that apply in Biacore™ systems, 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 characteristics or amount of ligand immobilized on the surface do not influence the transport rate.

What limits the observed binding?

In analysis, the observed rate of binding (i.e., the slope of the sensorgram) is determined by the relative magnitudes of the net biochemical interaction rate and the rate of mass transport. When the interaction is much faster than transport, the observed binding is limited by the transport processes. And when transport is fast and interaction is slow, the observed binding represents the interaction kinetics alone.

When the rates of the two processes are of similar orders of magnitude, the binding is determined by a combination of the two rate characteristics. The net biochemical interaction rate (see *Factors determining binding rates, on page 29*) 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 an injection – mass transport processes can be limiting at the beginning of the injection, while interaction limits the observed binding rate at later stages.



In a partially mass transport-limited situation, mass transport dominates at the beginning of the injection and interaction rate dominates later in the injection.

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 that support this measurement approach, this involves fitting the 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 is described by the equation below, where k_m is the mass transport coefficient and k_a and k_d are the interaction rate constants:

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

The rate of mass transport from bulk solution to the surface is described by:

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

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

As a general recommendation, you should make measurements at two or more widely separated flow rates (for example 5 and 100 µL/min). Then you can fit the data 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).

Mass transport in laminar flow systems

In the laminar flow conditions that apply in Biacore™ systems, the mass transport coefficient k_m is related to the analyte diffusion coefficient D by the expression:

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

Where: D is the diffusion coefficient of the analyte in m^2/s
f is the volumetric flow rate of liquid through the flow cell in m^3/s
h, w, and l are the flow cell dimensions (height, width, length in m)

The mass transport coefficient k is expressed in 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:

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

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

Diffusion coefficients for protein analytes

You can find values for the diffusion coefficient of many proteins in the literature. If you know the analyte's molecular properties, you can also calculate them by using the Diffusion Coefficient Calculator available on cytiva.com/biacoretools. 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 (η) of the solution, so that if you can find values for one set of conditions, you can easily calculate the corresponding values 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)
η is the viscosity of the solvent
subscript ref indicates reference conditions

If the experimental temperature differs from the reference value, you should correct the relative viscosity values for temperature. However, you can consider the viscosity of common physiological buffer solutions (containing 0.15 M salt and no major additives such as glycerol) to be equal to that of water – you rarely need to correct for buffer composition for these solutions.

Estimating diffusion coefficients from molecular properties

If there is no value available in the literature for the diffusion coefficient of the analyte you're studying, you can estimate a value from the molecular weight and shape factor, or *frictional ratio* according to the equation below. This is a semiempirical 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
η_{rel} is viscosity of the solvent relative to water at 20°C
(η_{rel} for water or buffer at 25° = 0.89)

Typically, values for globular proteins with a molecular weight around 100 000 Daltons are around 6×10^{-11} m^2/s at 25°C.

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 transfer to corresponding errors in the measured concentration. Underestimating the diffusion coefficient results in overestimating the concentration by the error factor raised to the power of 2/3 (this follows from the relationship between k_m and D, see *Technical aspects of calibration-free assays, on page 31*). For example, underestimating the diffusion coefficient by a factor of 2 will result in concentration values that are too high by a factor of about 1.6.

Researchers need to assess the reliability of measured diffusion coefficients reported in the literature on a case-by-case basis, based on the validity of the experimental measurements.

If the molecule is not globular and you don't know the frictional factor, your estimates of the diffusion coefficient from the molecular properties might be incorrect by a significant factor. Elongated and inflexible protein molecules can have frictional ratios as high as 10 or 20, so knowing the molecular shape is important for correct estimations.

Other factors like 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 mixed components binding to the sensor surface.

To evaluate calibration-free concentration measurements, you can calculate the mass transport coefficient k_m from the diffusion coefficient, and then convert it to the mass transport constant k_t (see *Mass transport in laminar flow systems, on page 32*). You'll also use the mass transport constant to fit the experimental data to the diffusion-controlled interaction model. Any uncertainties in the molecular weight and response-to-concentration conversion factor will affect the calculated concentration.

When assessing the reliability of your results, consider the following aspects:

- It is important to have 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. 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 you can assess the influence of flow rate on the binding rate.
- In general, your results are 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.
- Your experimental data should closely fit the interaction model you used to evaluate the concentration. If the fit is poor, the calculated concentration will be correspondingly unreliable.

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