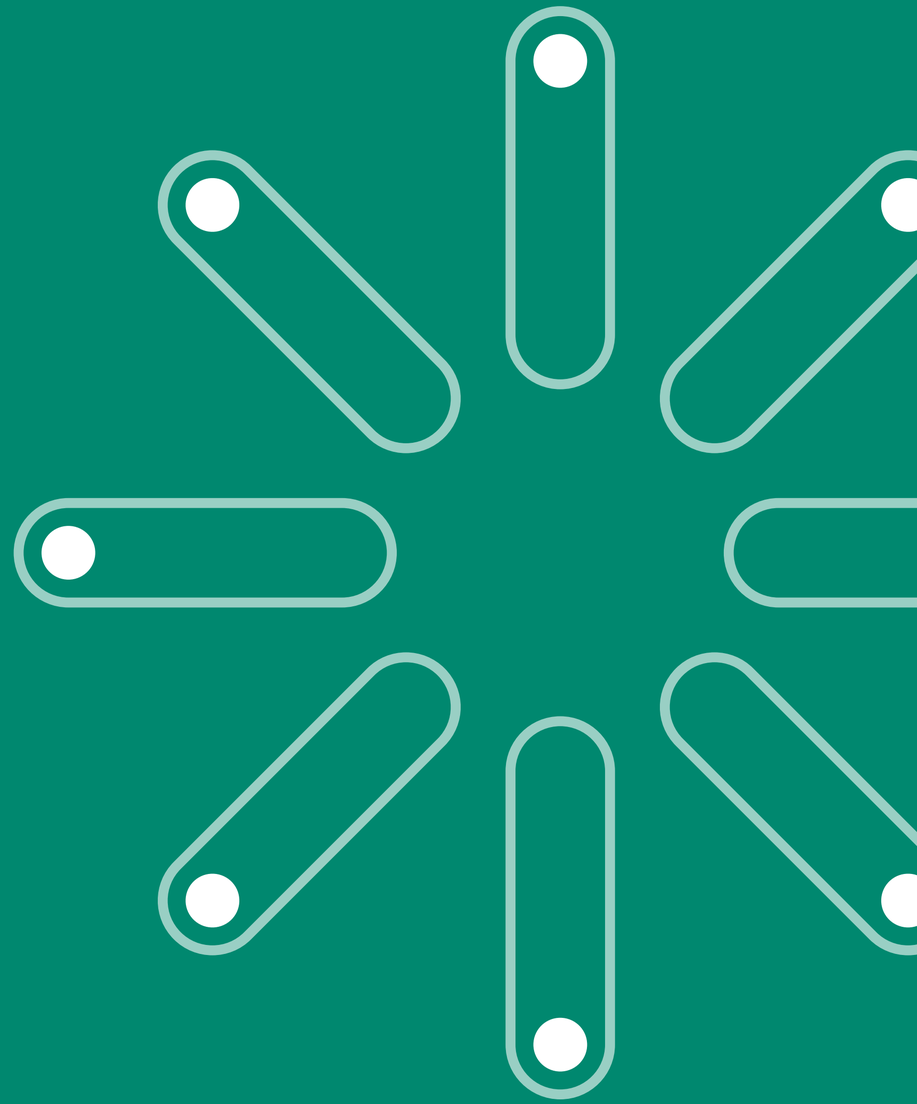
Biacore application guides

Fragment and small molecule screening with Biacore systems



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The primary goal of fragment and small molecule (low molecular weight, LMW) screening in pharmaceutical development is to identify candidate molecular structures for further development, based on their binding to selected target molecules. Response levels are low and organic solvents are often needed to maintain analyte solubility. Interactions are usually rapid and regeneration is not needed.

Screening often starts with a library of compounds, either commercial or proprietary, that may contain thousands or even millions of compounds. High throughput is therefore crucial in the initial screening stages. Selection of candidates for further development, particularly from fragment libraries, is seldom based on detailed evaluation of binding characteristics. As development work progresses, the number of candidate molecules is reduced and focus can be directed towards more detailed characterization.

Small molecule libraries can often consist of millions of compounds, while fragment libraries generally consist of thousands of structures. Small molecule affinities usually range from nM to µM while fragment affinities are usually in the range from high µM to mM range.

Terminology

Term	Meaning
Fragment	A small chemical structure (molecular weight typically 150 to 300) that may contribute to binding to a target molecule. In fragment-based drug discovery, fragments with relatively weak binding affinities are chemically combined to form larger structures with higher specificity and improved binding characteristics.
Small molecule	An organic compound (molecular weight typically several hundred Da) that may in itself be a potential drug candidate. Small molecules may be derived from natural sources or created by combination of fragments.

The borderline between fragments and small molecules is not well-defined: larger structures formed by combination of fragments eventually fall into the category of small molecules. Several techniques described in this Application guide can be used on both fragments and small molecules.

Assay formats

Screening for target binding is normally performed using a direct binding assay (DBA). The target is immobilized on the surface and the LMW compound or fragment is injected as analyte. Relatively high levels of target molecule are required to compensate for the intrinsically low responses obtained with low molecular weight analytes.

Competition assays such as inhibition in solution assay (ISA) or surface competition assay (SCA) can be used to obtain information on site specificity. Description of assay setup and evaluation for competition assays is outside the scope of this Application guide.

Support for fragment screening in several Biacore™ systems covers three types of screen, performed sequentially:

Step	Purpose
Clean screen	Eliminate "sticky" compounds from the fragment library. Sticky compounds remain on the surface after sample injection and can interfere with subsequent cycles.
Binding level screen	Identify potentially interesting fragments on the basis of levels of binding to the target
Affinity screen	Refine the fragment selection on the basis of estimated affinity to the target

Tips for fragment and LMW screening

- Use a direct binding assay
- Before starting the screen, estimate how much target you need to immobilize in order to obtain sufficient analyte response
- Confirm the activity of the immobilized target with a positive control compound
- Scout for and optimize buffer composition before starting the screen, particularly with respect to non-specific binding
- Choose the screening concentration according to the binding strength you want to detect (for example, use µM concentrations if you expect µM affinities). Using higher concentrations increases the risk of false positive results.
- Test different temperatures for the screen (for example, 25°C, 20°C and 15°C). Temperature may have a major impact on both binding characteristics and target protein stability.
- If you use DMSO, make sure all equipment (pipette tips, microplates, vials, filters, and so on) are compatible with organic solvents. Do not use polystyrene products with DMSO.
- Prepare negative controls in the same way as other samples. Do not use running buffer as a negative control.
- Make sure samples are thoroughly mixed when they are diluted from stock solutions. Poor mixing can give rise to unexpected sensorgram shapes with correspondingly deviant screening results.
- Run solvent correction samples, positive and negative controls at even intervals throughout the assay
- Use a data collection rate of 1 Hz for screening that will be evaluated from report points. Resolution of fast binding events is not required.
- Keep the Biacore instrument clean and well-maintained in order to minimize signal noise and disturbances

General considerations

Analyte properties

The following properties are important in the design and evaluation of fragment and LMW screening in Biacore systems:

- Fragments and small molecules in pharmaceutical development frequently show limited solubility in aqueous solutions. Addition of organic solvents (usually dimethyl sulfoxide, DMSO) is often needed to maintain solubility. See the separate guide Solvent correction: Principles and practice for further details.
- The low molecular weight of fragments and small molecules (typically 150 to 300 Da for fragments, a wider range for small molecules) means that responses in Biacore systems are intrinsically low. Regular cleaning and maintenance is essential for maximum system performance.

  Note: High responses can sometimes be observed from fragments that aggregate into larger structures.
- Binding to and dissociation from the target molecule is usually rapid
- Binding affinities are often weak, particularly for fragments where the affinity is typically in the high µM to mM range

Sensor surface preparation

Because of the inherently low responses from small molecules, together with the frequently low affinity, sensor surfaces for LMW and fragment screening are prepared with relatively high levels of ligand (typically 8000 to 10 000 RU for average-sized proteins). The theoretical binding capacity can be calculated from the ligand level and the relative sizes of ligand and analyte. For example, immobilizing 10 000 RU of a ligand with molecular weight 150 000 Da will give a theoretical binding capacity of 300/150 000*10 000 = 20 RU for an analyte with molecular weight 300 Da.

Buffers

The following table lists recommendations for buffers used in a screening experiment. If results are not satisfactory, be prepared to optimize buffer composition using buffer scouting experiments with a small set of analytes.

The composition of sample buffer and running buffer should be matched as closely as possible to minimize bulk refractive index effects. Small variations in salt and particularly DMSO concentration lead to variations in bulk refractive index contribution that may be of a similar order of magnitude to the expected analyte responses.

Component	Recommendations
Buffer substance	Phosphate buffers (10 to 50 mM) are generally recommended for work with small molecules and fragments. Organic buffer components such as HEPES can bind to the ligand and interfere with the detection of small organic compounds.
Ionic strength	Physiological ionic strength (150 mM monovalent cations, normally sodium) should be used to reduce non-specific binding of compounds to the sensor surface
Detergent	Inclusion of detergent (0.05% Surfactant P20) generally improves data quality by reducing drift and signal disturbances
DMSO	Running buffer and samples must contain the same concentration of DMSO. Recommended DMSO concentrations are 2% to 5%.
	Laboratory protocols for preparing buffers and samples with matching DMSO concentration are available from Cytiva

Sample preparation

Make sure that all material used for sample preparation is compatible with DMSO. Do not prepare samples in polystyrene microplates or vials: DMSO can extract components from the plastic and contaminate samples and buffers. Use polypropylene plates and vials. Mix all samples carefully and ensure that samples and running buffers are matched as closely as possible with respect to DMSO content to avoid large differences in bulk refractive index.

For best performance prepare all blank samples and negative controls as far as possible in the same way as samples. Prepare blanks and negative controls in separate wells in the microplate. Do not use running buffer as blanks or negative controls, and do not pool solutions. This will help to avoid systematic errors that may arise from different preparation methods. Detailed laboratory protocols for preparation of buffers and samples are available from Cytiva.

Clean screen

Purpose

Clean Screen helps to identify residual binding of fragments to the target molecule and/or the sensor surface. Fragment screening cycles do not normally include regeneration, and residual binding may impact subsequent cycles and mask weak but well-behaved binding of other fragments. Fragments that show residual binding can be removed from subsequent screening steps or placed last in the sample sequence.

Clean screen setup

Clean Screen is performed by injecting single concentrations of each fragment over reference and target surfaces. Additional surfaces may be included for control proteins, to reveal whether residual binding is promiscuous or specific to the target molecule.

The table below summarizes recommended experimental conditions.

Parameter	Value
Flow rate	30 µL/min
Contact time	10 to 30 s
Dissociation time	0 s
Fragment concentration	Typically 1 mM for all fragments
Regeneration	Not used
Molecular weights	Not used
Startup cycles	Include 1 to 3 startup cycles to equilibrate the system before injecting the first sample
Reference subtraction	Not used
Extra wash	Optional (50% DMSO recommended) Extra wash does not pass over the sensor surface
Solvent correction	Not used
Control samples	Not used

Note: Residual binding of one fragment is detected by an increased baseline level in the next cycle. This requires an extra blank cycle after the last sample in the screen. Some Biacore systems include the extra cycle automatically in **Clean screen** methods.

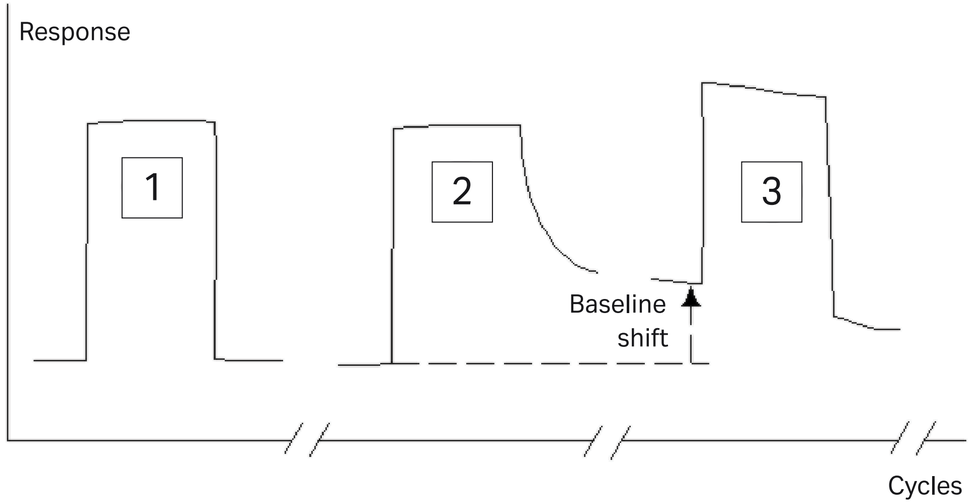
Clean screen evaluation principle

Evaluation of **Clean Screen** is based on the shift in absolute baseline response from one cycle to the next. The baseline shift for cycle **n** is calculated as

(baseline response in cycle **n+1**) **-** (baseline response in cycle **n**)

Residual binding is identified by a baseline shift above a chosen cut-off level. In the schematic illustration on the right, the higher baseline response in cycle 3 indicates residual binding in cycle 2.

Fragments that show residual binding to both the target and reference surface and to any control surfaces included in the screen are classed as general residual binders. Those that show residual binding to one surface but not the other are selective residual binders.



Clean screen evaluation workflow

In Biacore systems that support evaluation of **Clean screen**, follow the steps below to identify sticky fragments:

Step	Action
1	Prepare the **Clean screen** plot of baseline shift against cycle
2	Set a suitable cut-off response
3	Note the cycles where the baseline shift is above the cut-off. Use table functions and curve markers if these are available to help identify the cycles.
4	Examine the baseline plot and sensorgrams to establish the cause of the baseline shift. Stable baseline shifts do not indicate sticky fragments and can be ignored.

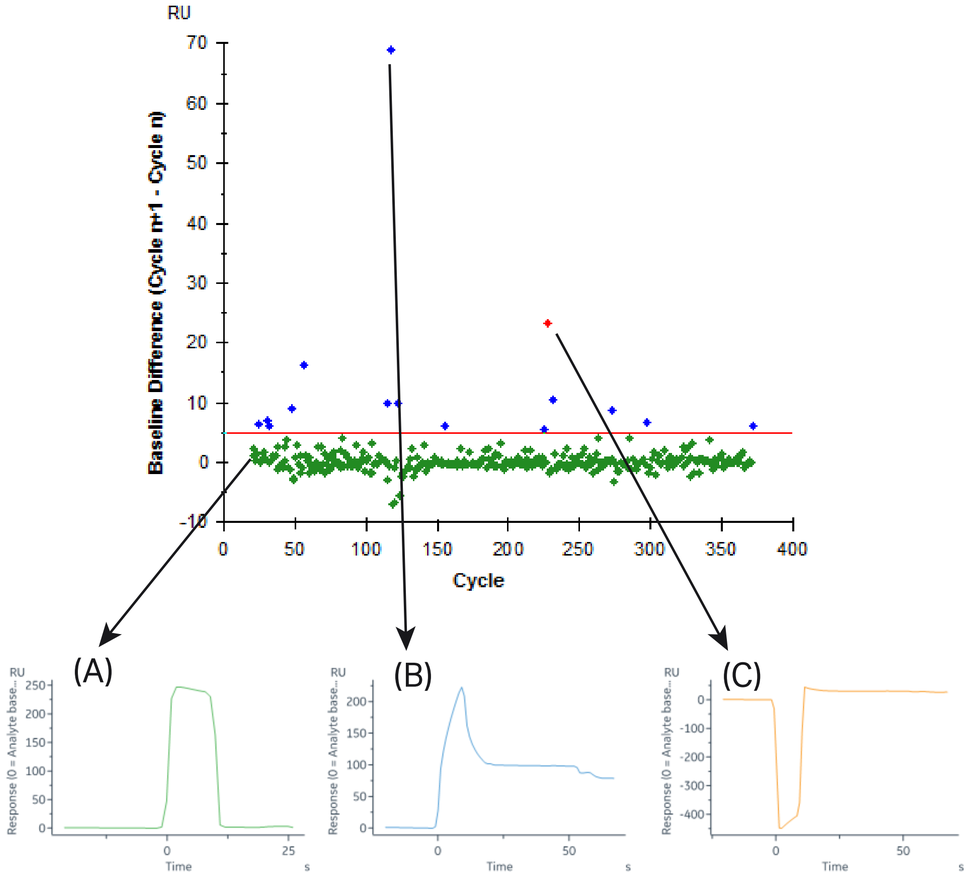
In Biacore systems that do not support **Clean screen** evaluation directly, examine the baseline plot and sensorgrams to identify sticky fragments, or export the binding data to a third-party application (for example, Microsoft® Excel®) and construct a baseline shift plot.

Clean screen results

An example of the results from a **Clean Screen** with sensorgrams corresponding to selected points are shown to the right.

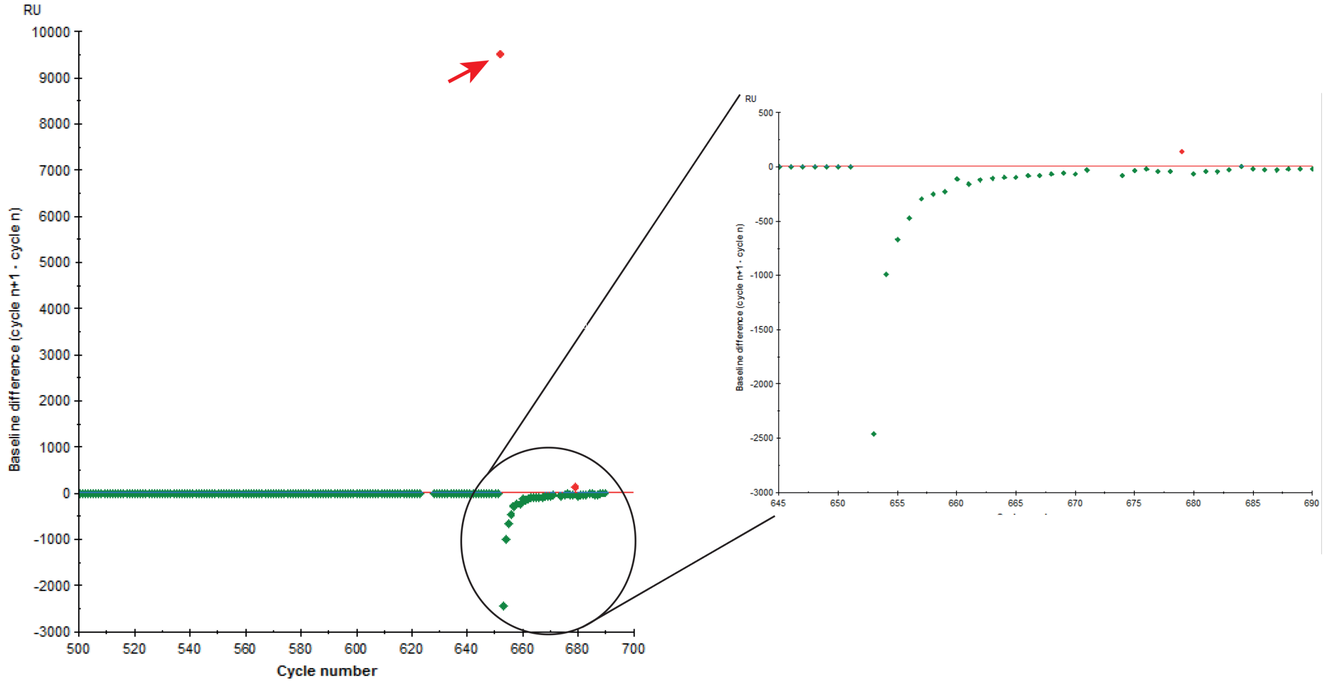
- Sensorgram A shows a normal sample with no residual binding
- Sensorgrams B and C show samples with residual binding that shows little dissociation during the injection

Note: The negative relative response in sensorgram C represents bulk refractive index and does not affect the interpretation of the **Clean Screen**.



Effects of residual binding

An example of extreme consequences of residual binding is shown below. In this case, the single “sticky” fragment (indicated by the arrow) shows a very high residual binding (above 9000 RU), probably as a result of aggregate formation or precipitation. The residual binding carries over at least 10 subsequent cycles.



Binding level screen

Purpose

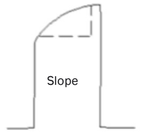
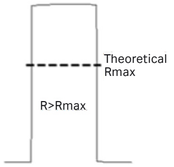
The purpose of **Binding level screen** is to identify binders to the target and to find and exclude fragments with atypical binding behavior. Fragments at this stage are identified by both binding response and by curve shape which gives insight into the binding characteristics of the fragments.

Note: **Binding level screen** for fragments does not usually give confident identification of binders, and is used primarily to reduce the number of fragments chosen for further development.

Binding behavior

Both binding and dissociation are usually rapid for fragments, giving “square-wave” sensorgrams with no resolution of association or dissociation.

Atypical binding behavior is however relatively common, and is identified from the shape of the sensorgrams. Three kinds of atypical binding are recognized:

Name	Description	Sensorgram appearance
Slope	Response increases significantly during sample injection instead of the normally expected rapid binding to a steady-state level. **Slope** is determined from the average slope of the sensorgram between the report points **binding_early** (shortly after injection start) and **binding** or **binding_late** (shortly before injection end).	
Slow diss	The compound does not dissociate immediately after the end of the sample injection. **Slow diss** is determined from the relative response above baseline for the report point **stability** (shortly after the end of the injection).	
R>Rmax	The maximum response reached during sample injection is higher than the theoretically expected value, calculated from the amount of ligand on the surface and the molecular weights of the ligand and fragment.	

Binding level screen setup

Binding level screen is usually performed by injecting a single fragment concentration over active and reference surfaces. Solvent correction cycles and positive and negative controls should be included.

The table on the right summarizes recommended experimental conditions.

Parameter	Value
Flow rate	30 µL/min
Contact time	20 to 30 s
Dissociation time	0 to 15 s
Fragment concentration	Typically 1 mM for all fragments
Regeneration	Not used
Extra wash	Optional (50% DMSO recommended) Extra wash does not pass over the sensor surface
Molecular weights	In Biacore systems with dedicated support for **Binding level screen**, molecular weight of both immobilized target and fragments are required for full evaluation functionality
Startup cycles	Include 1 to 3 startup cycles to equilibrate the system before injecting the first sample
Solvent correction	Repeated at regular intervals. The recommended interval differs in different systems. See the system-specific documentation for details.
Positive controls	Known binder (not necessarily a fragment) that dissociates rapidly
	Repeated at regular intervals. The recommended interval differs in different systems. See the system-specific documentation for details.
Negative controls	Prepare negative controls in the same way as samples, using buffer instead of fragment. Do not use running buffer.
	Repeated at regular intervals. The recommended interval differs in different systems. See the system-specific documentation for details.
Blank cycles	Repeated at regular intervals. The recommended interval differs in different systems. See the system-specific documentation for details.
	Distinguish between blank cycles and negative controls. The cycles have different purposes even if they can use the same sample.

Binding level screen evaluation

Evaluation of **Binding level screen** is based on report point values and binding behavior (see *Binding behavior*, on page 12) after reference subtraction and solvent correction.

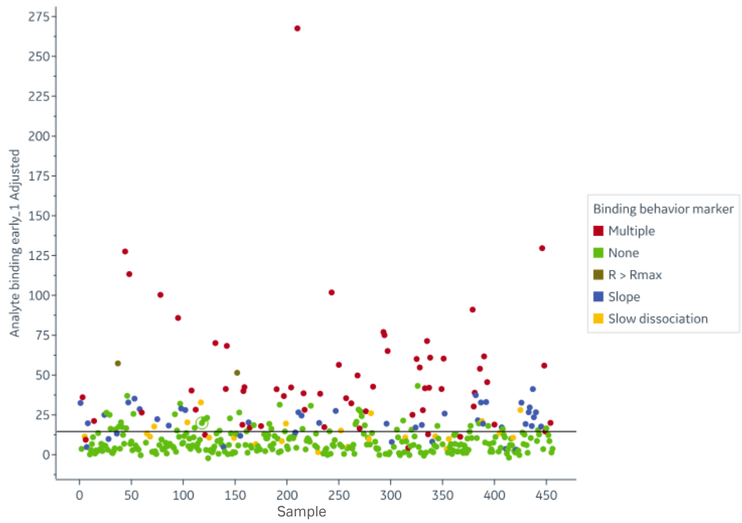
Fragments usually bind rapidly to primary sites, while aggregative binding types are significantly slower. Contribution of slower binding to the screening results are reduced by using a **binding_early** report point, placed shortly after the start of the injection. Reference subtraction and solvent correction eliminate in principle bulk refractive index contributions to the **binding_early** response.

Follow the steps on the right, as far as they are supported in the Biacore system used, to evaluate a **Binding level screen** for fragments. Refer to the documentation for your Biacore system for detailed description of the evaluation steps.

Step	Action
1	Apply solvent correction
2	Prepare a reference-subtracted, solvent corrected plot of **binding_early** against cycle
3	Apply blank subtraction. Examine the blank cycle sensorgrams carefully and exclude any cycles with significant disturbances.
4	Apply molecular weight adjustment to compensate for differences in fragment size
5	Apply adjustment for controls, to compensate for changes in surface activity and negative control responses during the course of the experiment
6	Apply median filtering if desired, to compensate for any remaining systematic disturbances
7	Set a cut-off to select the fragments with the highest binding. Frequently, the cut-off level will be chosen on the basis of the number of fragments above cut-off.
8	Normally, fragments with binding behavior markers (see *Binding behavior*, on page 12) will be excluded from further screening experiments

Binding level screen example

An example of a binding level screen is shown on the right. The plot legend identifies binding behavior.



Affinity screen

Purpose

The purpose of **Affinity Screen** is to verify target binding and to obtain an estimate of steady-state affinities of fragments selected in previous screens. Expected affinities for fragments are commonly in the high micromolar to millimolar range.

Constant Rmax

For reliable determination of binding affinity using a standard model with variable Rmax, the highest sample concentration analyzed should in general be at least twice the equilibrium dissociation constant (K_D) value. This guideline can frequently not be met with fragments, because of low solubility and affinities in the mM range.

An alternative fitting model **Steady state affinity (constant Rmax)**, provided in some Biacore systems, uses a constant value for R_{max} to force an asymptote on the fitted curve. The R_{max} value is best obtained from injection of a known binder, called the **Rmax control**, at saturating concentration or from kinetic or affinity fitting to a concentration series of the Rmax control. The measured value expressed in RU/100 Da is entered in the software, and the R_{max} for each sample is calculated from this value and the molecular weight of the fragment. The assumption is made that both R_{max} control and all fragments share the same binding stoichiometry.

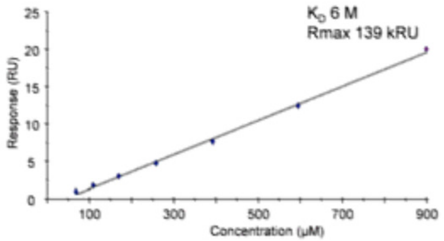
Example:
The table below shows how the constant R_{max} value is calculated for two hypothetical samples.

Sample	MW (Da)	Measured R_{max} (RU)	RU/100Da	Calculated R_{max} (RU)
Rmax control	600	40	15	-
Fragment1	200	-	-	30
Fragment2	150	-	-	22.5

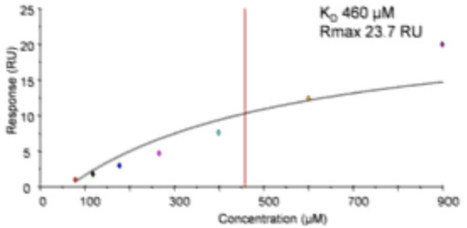
As a last resort, if there is no suitable R_{max} control available, a theoretical R_{max} can be calculated using the immobilization level of the ligand and the molecular weights of the ligand and a fictive control compound. This approach is less reliable, but can be used for comparing affinities determined on the same, stable surface.

Example

An example of steady state affinity fitting with variable and constant Rmax is shown below.



Data fitted with variable R_{max}.



The same data fitted with constant R_{max}.

Affinity screen setup

For **Affinity screen**, a series of 7 to 8 fragment concentrations and one blank is injected over the target surface, and the binding levels are analyzed by fitting to a 1:1 steady state affinity model.

Positive and negative control samples are recommended to allow compensation for changes in surface activity during the course of the screen. Blank samples are included to allow blank subtraction. An R_{max} control sample is recommended.

The table on the right summarizes recommended experimental conditions.

Parameter	Value
Flow rate	30 µL/min
Contact time	30 s
Dissociation time	30 s
Fragment concentration	Typically 1.5-fold dilution series from 1000 to 50 µM
Regeneration	Not used
Extra wash	Optional (50% DMSO recommended) Extra wash does not pass over the sensor surface
Molecular weights	In Biacore systems with dedicated support for **Affinity screen**, molecular weight of both immobilized target and fragments are required for full evaluation functionality
Startup cycles	Include 1 to 3 startup cycles to equilibrate the system before injecting the first sample
Solvent correction	Repeated at regular intervals. The recommended interval differs in different systems. See the system-specific documentation for details.
Positive controls	Known binder (not necessarily a fragment) that dissociates rapidly
	Repeated at regular intervals. The recommended interval differs in different systems. See the system-specific documentation for details.
Negative controls	Prepare negative controls in the same way as samples, using buffer instead of fragment. Do not use running buffer.
	Repeated at regular intervals. The recommended interval differs in different systems. See the system-specific documentation for details.
Rmax control	Included or determined in a separate experiment
Blank cycles	Repeated at regular intervals. The recommended interval differs in different systems. See the system-specific documentation for details.
	Distinguish between blank cycles and negative controls. The cycles have different purposes even if they can use the same sample.

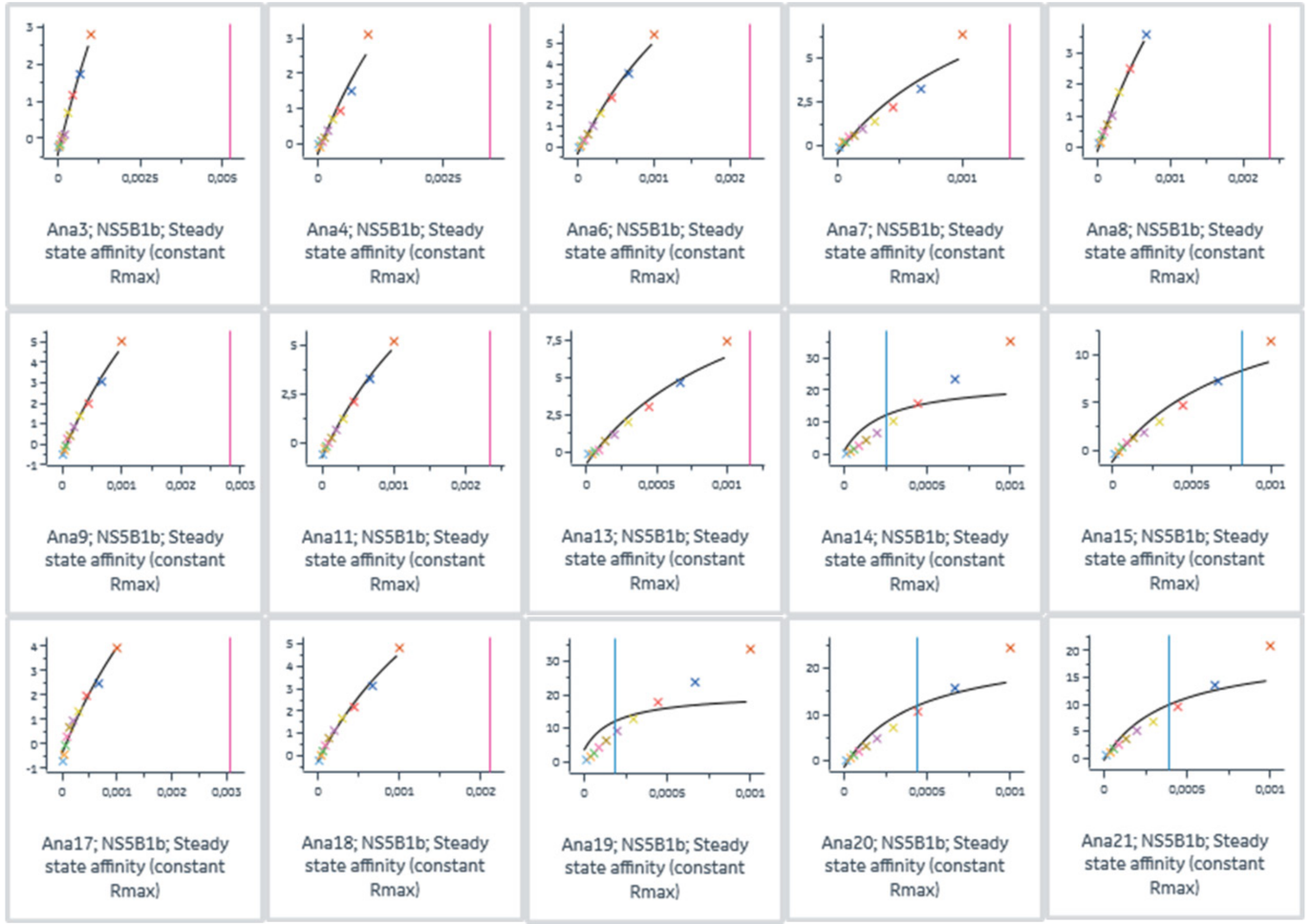
Affinity screen evaluation

To reduce the effect of slow aggregative binding on the affinity determination, use binding levels shortly after the start of the injection as steady-state levels.

Fit the binding level data to a steady state affinity model with constant R_{max}.

Select fragments for further development based on the reported affinities.

Several Biacore systems present the results of **Affinity screen** as a graphical thumbnail overview (the illustration on the right is taken from Biacore 8K) with a table of data including affinity constants.



Small molecule screens

Purpose

The purpose of LMW (small molecule) screening is to find and characterize small molecules with target-specific and non-promiscuous binding.

Small molecule screen setup

LMW screening is usually performed as a single-concentration screen of a set of compounds.

The table on the right summarizes recommended experimental conditions.

Parameter	Value
Flow rate	30 µL/min
Contact time	30 s to 60 s
Dissociation time	60 s
Sample concentration	Recommended starting value 30 µM
	May need to be adjusted according to the expected binding affinity. Use the same order of magnitude as the expected affinity (for example, if µM affinities are expected, use µM concentrations.
Regeneration	Not used
Carry-over injection	Included to detect “sticky” compounds
Extra wash	Optional (50% DMSO recommended) Extra wash does not pass over the sensor surface
Molecular weights	Required for adjusting response levels for molecular size
Startup cycles	Include 1 to 3 startup cycles to equilibrate the system before injecting the first sample
Solvent correction	Repeated at regular intervals. The recommended interval differs in different systems. See the system-specific documentation for details.
Positive controls	Known binder that dissociates rapidly
	Repeated at regular intervals. The recommended interval differs in different systems. See the system-specific documentation for details.
Negative controls	Prepare negative controls in the same way as samples, using buffer instead of compound. Do not use running buffer.
	Repeated at regular intervals. The recommended interval differs in different systems. See the system-specific documentation for details.
Blank cycles	Repeated at regular intervals. The recommended interval differs in different systems. See the system-specific documentation for details.
	Distinguish between blank cycles and negative controls. The cycles have different purposes even if they can use the same sample.

Small molecule screen evaluation

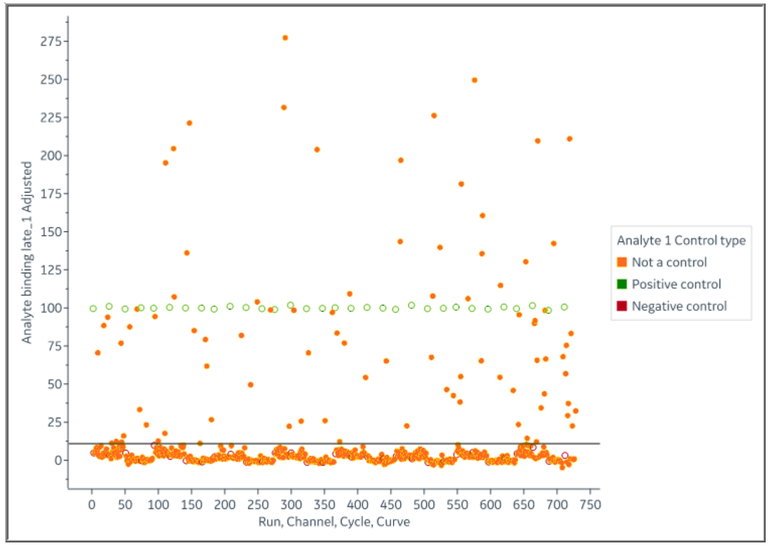
LMW screens are evaluated by plotting and comparing binding responses, normally using the report point **binding_late** (set shortly before the end of the sample injection).

Binding levels may be ranked in relation to control sample responses or custom ranking boundaries.

Follow the steps below as required to evaluate an LMW screen:

Step	Action
1	Apply solvent correction
2	Prepare a reference-subtracted, solvent corrected plot of **binding_late** against cycle
3	Apply blank subtraction. Examine the blank cycle sensorgrams carefully and exclude any cycles with significant disturbances.
4	Apply molecular weight adjustment to compensate for differences in small molecule size
5	Apply adjustment for controls, to compensate for changes in surface activity and negative control responses during the course of the experiment
6	Apply median filtering if desired, to compensate for any remaining systematic disturbances
7	Set a cut-off to select the compounds with the highest binding or use ranking boundaries to define multiple levels of binding (such as **low, medium, high**)

An example of an LMW screen with a cut-off level set to the average negative control response plus 3 standard deviations is shown below.



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