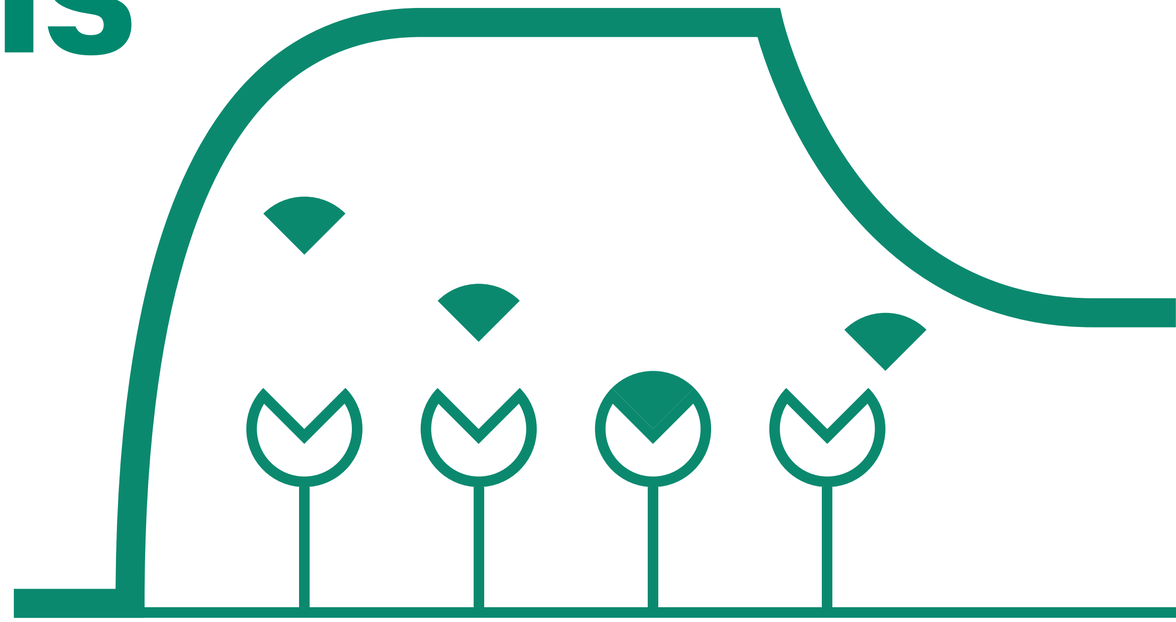
Biacore application guides

Competition screening with Biacore systems



cytiva

Introduction

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. Compound libraries are often very large, and early screening focuses on surveying libraries, to identify and prioritize potential binders (see the Biacore™ application guide *Fragment and small molecule screening with Biacore systems*). Competition screening typically follows the early screening steps and focuses on a smaller number of binders.

In Biacore systems, competition screening is performed by monitoring binding in the presence and absence of a high concentration of competitor that is known to bind to a site of interest on the target molecule. Examination of the binding levels reveals whether binding of the competitor and test substance to the target is exclusive or additive. Exclusive binding, where the competitor blocks binding of the sample, indicate that the sample and competitor bind to the same or sterically interfering sites. Additive binding indicates that the sample and competitor bind to separate (allosteric) sites.

Evaluation software included with Biacore systems does not currently provide any dedicated tool for evaluation of competition screens. Tools for cut-off and ranking in response level plots are generally sufficient.

Scope of this application guide

This application guide provides general guidelines for competition screening of fragments and small molecules.

This guide is based on work performed using a Biacore S200 system, but may be expected to apply in general terms to screening experiments on other Biacore systems. Independent considerations may be found in *Perspicace et al (2009), Journal of Biomolecular Screening, **14**, 337-349* and *Giannetti et al (2014), Journal of Medical Chemistry, **57**, 770-792.*

Terminology

Term	Meaning
Fragment[1]	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[1]	An organic compound (molecular weight typically several hundred) that may be a potential drug candidate. Small molecules may be derived from natural sources or created by a combination of fragments.
Competitor	Site-specific competitor with known molecular weight and affinity
Sample mix	Assay format for competition screen where each sample is injected twice during a single run: once alone and once in a mixture with the competitor
Buffer mix	Assay format for competition screen that uses two separate runs. One run uses samples in running buffer without competitor, and the other includes competitor in both samples and running buffer.
A-B-A	Assay format for competition screen that uses the A-B-A injection type to change the buffer conditions temporarily in the flow cell. One injection is performed with competitor in the flanking solution and sample, and another without competitor.

[1] 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.

Tips for competition screening

- Choice of assay format depends primarily on availability of competitor and affinity of its binding to the target molecule
- Run competition assays on subsets of libraries. The assays are fairly time-consuming.
- Use a sufficiently high competitor concentration to block the target site completely if possible. Interpretation of the results is simpler if the site is completely blocked.
- 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.
- Expected binding responses for fragments are intrinsically low due to small size and weak binding. It is important to work carefully to keep experimental noise to a minimum.
- In single run assay formats, run samples with and without competitor in consecutive cycles. This will simplify evaluation.
- Run solvent correction samples, positive and negative controls at regular intervals throughout the assay
- For data consistency, use the same report point to evaluate binding with and without competitor
- Keep the Biacore instrument clean and well-maintained in order to minimize signal noise and disturbances

Assay formats

Introduction

Competition screening may be run in one of two general ways:

- A binding level screen is performed on the sample library to identify hits (see the Biacore application guide *Fragment and small molecule screening with Biacore systems*). A separate competition screen is then performed on the hits. This involves comparison of binding data from two separate runs, which may or may not have been run on the same occasion.
- A combined binding level and competition screen is performed on the whole sample library, to identify hits and characterize site-specificity in a single run

The competition screen itself can be set up in different formats, referred to as *Sample mix, Buffer mix* and *A-B-A*, as described on the following page.

Sample mix

Competitor is mixed with sample outside the Biacore instrument. Sample with and without competitor may be analyzed in separate runs or in separate cycles within the same run.

Buffer mix

Competitor is mixed with both running buffer and sample, and is thus maintained at a constant concentration throughout the run. This approach necessarily requires that binding without competitor is determined in a separate run. The runs may be combined into one unattended operation in Biacore systems that include a buffer selector valve.

A-B-A

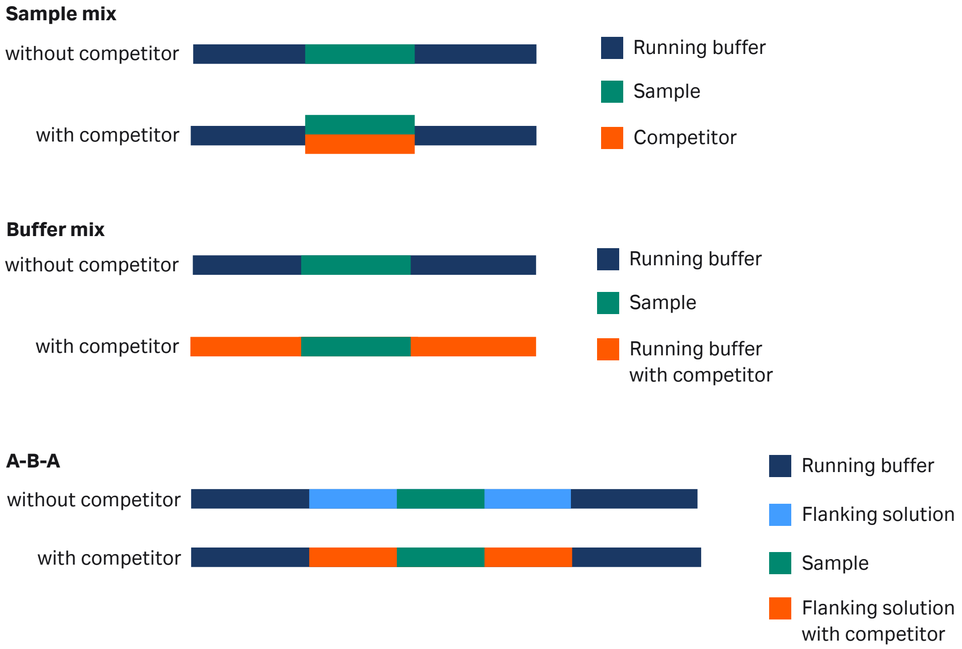
This approach uses the A-B-A injection type (available in some Biacore systems) to change the buffer conditions temporarily in the flow cell, by injecting a flanking solution before and after the sample. One injection is performed with competitor in the flanking solution and sample, and another without competitor.

Choice of assay format

The three formats differ primarily in suitability in relation to competitor affinity and in consumption of competitor:

Assay format	Competitor affinity (K_D)	Competitor consumption
Sample mix	Medium (µM)	Low
Buffer mix	Medium to high (µM to nM)	High
A-B-A	Medium (µM)	Medium

Sample mix and A-B-A formats are less suitable if bound competitor is difficult to remove from the surface between cycles.



Assay development and 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 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 form larger aggregates.
- Binding to and dissociation from the target molecule is usually rapid for fragments and many small molecules
- 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.

Reference surface

Reference subtraction is important for assays where the measurements are taken during the sample injection. For most screening applications, an unmodified surface is acceptabe as a reference surface.

Buffers

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.

For more buffer recommendations, see the Biacore application guide *Fragment and small molecule screening with Biacore systems*.

Control samples

Control samples are required to establish cut-off levels for evaluating the results:

Control	Description
Positive control	Known binder to the same site as the competitor. Used to establish the cut-off for binding to the target in the presence of competitor. Ideally, the affinity of the positive control for the target molecule should be known, to simplify estimation of suitable sample and competitor concentrations (see *Sample and competitor concentration,* on page 10).
Negative control	Known non-binder or buffer. Used to establish the cut-off for identifying binders in the absence of competitor. Prepare negative controls in the same way as samples. Do not simply use running buffer.

Report points in A-B-A format

The default report point settings for A-B-A injections are inappropriate for competition screening in two respects. Depending on the Biacore system used, the default report points may be adjusted in Method Builder or in the evaluation software.

- Sample binding is measured using a report point set just before the end of the sample (solution B) injection. For fragment screening work, it is more suitable to use a point just after the start of the injection (see the Biacore application guide *Fragment and small molecule screening with Biacore systems).*

Solution:

Add a new report point (***binding_early***) set to 5 s after the start of the B injection.

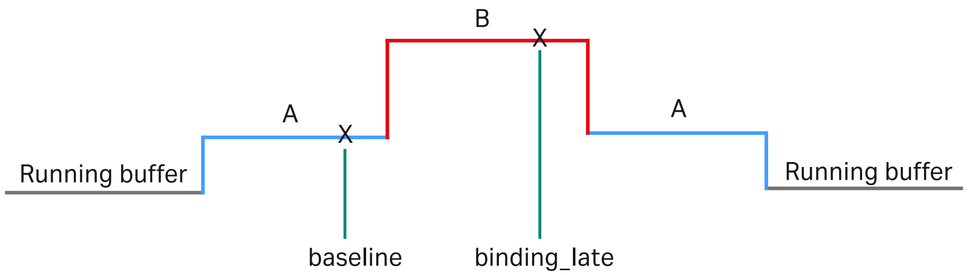
- The response for sample binding is calculated relative to a baseline in the flanking solution (A). While this gives a direct measure of sample binding in the presence of competitor, solvent correction factors, which are determined relative to running buffer, will be incorrect.

Solution:

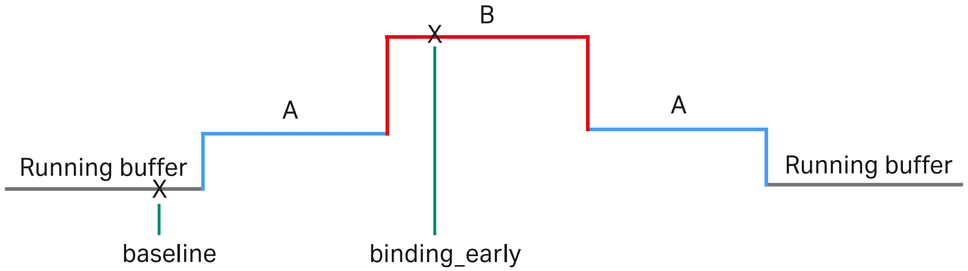
Move the baseline report point to a position 10 s before the start of the flanking solution (A) injection. Calculate binding levels relative to this new baseline.

These adjustments are illustrated schematically to the right.

Default settings



Adjusted settings



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.

Sample and competitor concentration

Because of the inherently low responses and the often low affinity of fragments and small molecules, high sample concentrations are required for detectable binding site occupancy. As a general recommendation, use as high a concentration as possible within the limits of the solubility of the sample compounds.

The competitor concentration should be sufficient to completely block the active site(s) on the target molecule. If the active site is not completely blocked, samples that bind to the same site will be able to bind to some extent even in the presence of inhibitor. A guide to suitable competitor concentrations can be obtained from calculations of fractional occupancy as described on the following page.

Fractional occupancy

The equations below describe fractional occupancies of a single site in terms of affinities and concentrations of two simultaneous binders A and B.

$$FO_A = \frac{1}{1+\frac{KD_A}{C_A}\left(1+\frac{C_B}{KD_B}\right)} \qquad FO_B = \frac{1}{1+\frac{KD_B}{C_B}\left(1+\frac{C_A}{KD_A}\right)}$$

For confident determination of exclusive binding, the fractional occupancy by the competitor is recommended to be 0.90 or greater.

Symbol	Meaning
FO_A, FO_B	Fractional occupancy of the site by binders A and B respectively
KD_A, KD_B	Dissociation equilibrium constants (K_D) for A and B respectively
C_A, C_B	Concentrations of A and B respectively

Example:

The table below shows the calculated fractional occupancy for 100 µM competitor (K_D 0.03 µM) in the presence of sample. The positive control (K_D 3 µM) at a concentration of 200 µM is blocked to 98% by the competitor. Samples with equilibrium dissociation constants (K_D) 1.5 µM or higher at concentrations up to 500 µM give satisfactory expected fractional occupancy by the competitor of 0.90 or higher.

Substance	K_D (µM)	Concentration (µM)	Fractional occupancy for competitor
Competitor	0.03	100	–
Positive control	3	200	0.98
Sample	> 1.5	< 500	> 0.90

Run settings

The table below shows the run settings found to be satisfactory in exploratory work with Biacore S200 at Cytiva, and can provide a starting point for optimization of settings for other situations.

Parameter	Sample mix	Buffer mix	ABA
Data collection (Hz)	1	1	1
Flow rate (µL/min)	30	30	30
Injection type	***Low sample consumption*** [1]	***Low sample consumption*** [1]	ABA
Injection time(s)			
Sample	60	60	60
Flanking solution	–	–	60
Extra wash	50% DMSO	50% DMSO	50% DMSO
Carry-over control	Yes	Yes	Yes

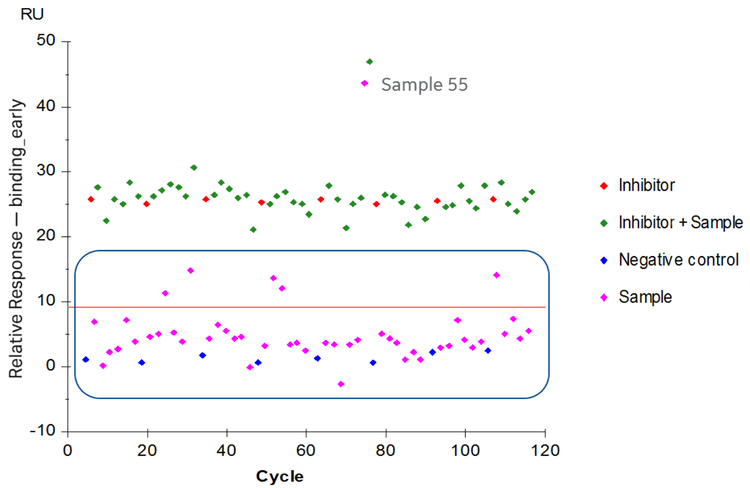
[1] In Biacore S200, use the injection type ***Binding level screen.***

Evaluation principles

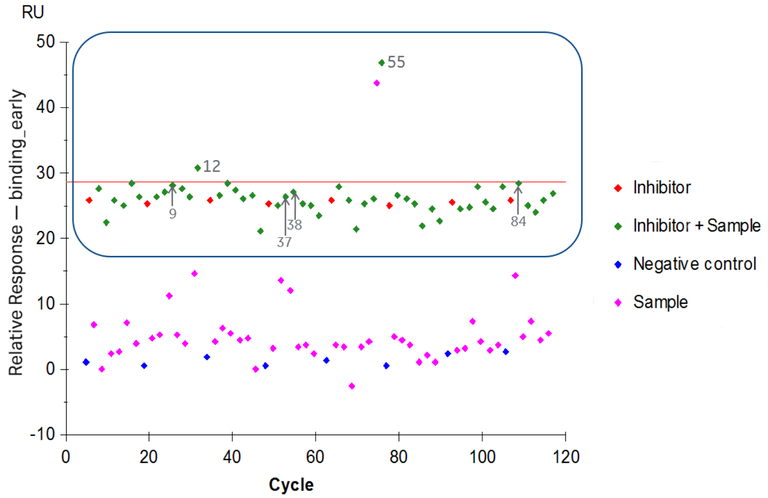
Evaluation workflow

In simple terms, evaluation of competition screening can be summarized as follows. Illustrations are taken from a combined binding level and competition screen using the **Sample mix** format.

Step	Action
1	Prepare a plot of response at an appropriate report point, arranged so that responses for each sample with and without competitor are readily identified (for example, placed beside each other).
2	**Examine the responses without competitor:** Set a cut-off boundary at the average negative control response plus a suitable number of standard deviations. Samples without competitor that give responses below this cutoff are regarded as non-binders and may be discarded. Filter these points out of the plot if the software supports this. **Example:**



Sample 55 gives anomalously high responses and can probably be discarded.

Step	Action
3	For the remaining points, set a new cut-off boundary at the average competitor response plus a suitable number of standard deviations. Samples above this cut-off show simultaneous binding of sample and competitor, indicating that the sample and competitor bind to separate sites. The remaining samples are potential binders to the same site as the competitor.  Binding of sample 12 is not completely eliminated by the presence of competitor. This sample can tentatively be identified as a separate-site binder.

Identifying cycles

To simplify side-by-side display of screening data with and without competitor, it may be valuable to identify the respective cycles by keywords, added as user-defined variables in the run method or as keywords in the evaluation session.

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