Compound- and fragment-based drug discovery and development using Biacore™ and MicroCal™ systems
Analytical tools for affinity-based, label-free drug discovery and development

Drug discovery requires identification and validation of target protein(s). Compounds with a potential to selectively bind the target are then screened for medicinal chemistry optimization programs. Once compounds are identified, their interactions with relevant targets are studied in detail to confirm and characterize the interactions quantitatively and to provide information on the binding mechanisms. Label-free biophysical methods such as those performed on Biacore and MicroCal systems are increasingly being adopted to achieve deeper insights into the interaction process. More accurate predictions of drug efficacy during the discovery phase can reduce late-stage attrition rates.

GE Healthcare Life Sciences provides instruments for both binding kinetics (Biacore systems) and thermodynamics (MicroCal systems). Kinetic characterization provides information about the rate of complex formation and its stability, whereas studies of interaction thermodynamics are required to resolve and understand the energetics of binding.

Label-free interaction analysis in compound discovery and development enable:

- Validation of the quality of the target protein
- Confident selection of candidates based on direct label-free binding data: selectivity, stoichiometry, affinity, kinetics, and thermodynamics
- Optimization of drug candidates for improved efficacy and safety through increased understanding of mechanism of action and pharmacokinetics and pharmacodynamics
- Using kinetic and thermodynamic data as key indicators for lead optimization by measuring residence time and enthalpic efficiency

This white paper gives examples of how label-free biophysical assays provide more information on compound-target interactions and how this information can be used to predict compound efficacy.

![Fig 1. A schematic overview of the compound discovery and development process supported by Biacore and MicroCal systems, from target validation to early ADME studies.](image-url)
Well-characterized drug targets give optimal assay performance and enable compound identification

A target-based drug discovery approach demands firstly the identification of a biological target with a causative activity related to the progression of a disease. Secondly, this protein has to be expressed and purified, and the quality has to be controlled. Thirdly, a therapeutic agent that modulates the activity of this target with limited or no adverse effects needs to be developed. Target identification is performed using various technologies such as genetic screens, small-interfering RNAs, and gene expression profiling (1).

In assay development and target validation, the label-free technologies, surface plasmon resonance (SPR) and microcalorimetry, can be used to answer a range of questions. Differential scanning calorimetry (DSC) addresses issues such as whether the target is properly folded, homogeneous and thermally stable.

The stoichiometric data extracted from isothermal calorimetry (ITC) experiments demonstrate the fraction of the target protein that is competent for binding to the lead compound. SPR, on the other hand, is used to indicate whether the protein binds the substrate and/or positive control with the “right” interaction kinetics as well as to demonstrate the activity level of the target protein.

Is my protein active?

In the following example, ITC is used to test the activity level of two batches of a target protein intended to be used as a high-throughput screening (HTS) assay reagent. In both cases, the protein batches were titrated with a reference compound that has a known 1:1 binding to the protein. Figure 2A shows the heat evolved as a function of molar ratio when the target protein from production batch 1 is titrated with the reference compound. The number of binding sites values was shown to be 1.05, which indicates that essentially all of the protein is active. Figure 2B shows the reference compound titrated into target protein from production batch 2. The binding site value of 0.23 indicates that the protein target is not fully functional and only 23% is active. Based on this information, it is very questionable if this second batch of target protein should be used in the HTS assay.

![Figure 2. Stoichiometry of binding is an important parameter extracted from ITC-experiments and distinguishes (A) pure protein from (B) badly folded, inactive protein.](image-url)
Deeper insights into secondary screening

HTS is still used for finding lead compounds with the potential to become therapeutic agents. In the pharmaceutical industry, SPR is heavily used as a secondary screen of thousands of HTS hits for elimination of false positives and promiscuous binders (2).

Structure-based biophysical drug design and virtual ligand screening generate new information and can potentially reduce the number of compounds that need to be evaluated. Focused and directed libraries have thus become popular alternatives to full libraries. Together with structural information and in silico methods, SPR is also used for identification of false negatives and for characterization of hits identified by structure-activity relationships (SAR) via catalogue approaches.

Fragment-based drug design is increasingly used to identify suitable chemical scaffolds in drug discovery. This approach is also structure-based and tries to identify very small compounds as initial anchoring points in different binding sites of the target protein. Both catalytic site and allosteric site binders can be found for both active and inactive forms of the target protein as no substrate is needed in biophysical, direct binding assays. Fragment-based drug design increases the possibility to cover a relatively large proportion of the chemical space by screening a smaller number of compounds; thousands instead of hundred of thousands to millions. Today, a combination of structural and functional binding information is used to identify promising fragments and it is crucial to perform an accurate and rapid prioritization of these fragments for further analysis and development. Biacore 4000 uses only microgram quantities of proteins for screening thousands of fragments (Fig 3).

Screening of fragments or directed libraries using SPR

Fragment screening presents several technical challenges. Biophysical methods such as X-ray crystallography and nuclear magnetic resonance (NMR) consume large amounts of target protein. The low affinities exhibited by fragments, typically in the mM range, require high sample concentrations with associated solubility and nonspecific binding issues. It is also difficult to measure binding affinities with these methods. SPR-based biosensors are attractive for this application due to low target consumption, high information content, and high-quality interaction data. The throughput of this technique is also several orders of magnitude higher than X-ray crystallography and NMR. Biacore 4000 can rapidly screen an entire fragment library against the target and reference proteins to remove compounds forming aggregates and other promiscuous binders with solubility problems (Fig 4 and 5). After this initial clean-up step, fragments are examined at a single concentration against multiple immobilized proteins (targets, blank reference, and additional proteins as selectivity references) to identify true binders, all in one assay. Binding site selectivity can be assessed by using target protein modified either by mutation or by covalent chemical blocking of the active site as an in-line reference together with the wild type protein. It is also possible to identify binding sites by running a competition assay and comparing the binding signal with and without a binding site-defining reference compound. With throughput sufficient for fragment and directed libraries, the methodology, data evaluation features, and enhanced sample data import and export functionality of Biacore 4000 are indispensable for screening directed libraries and for fragment based drug design.

**Fig 3.** The capacity of Biacore 4000 allows screening of fragment libraries using concentration series. A systematic increase in signal as function of increased concentration validates binding.

**Fig 4.** An optimal workflow of fragment screening has been implemented into the system and software of Biacore 4000. The same workflow is also well suited for secondary screening of hits from HTS. A complete screen and hit characterization according to this workflow takes approx. 1–2 weeks for a library of 2000 fragments.
Hit profiling for improved prediction of drug efficacy

Hit profiling is a very important step in the process of small molecule drug discovery. At this point, critical decisions are made determining which compounds to move forward to lead optimization. Choosing the best candidate at this stage can have a huge impact on reducing the attrition rate of drug candidates.

Biophysical assays such as SPR and ITC resolve affinity into binding rate constants (giving information on parameters such as residence time) and binding energetics (giving information on enthalpy) and thus provide a quantitative picture of how the compounds from different scaffold classes interact with the target molecule. This improved resolution makes it possible to better judge the biological potential of the lead series as the relevance of pharmacokinetic data is strongly correlated with binding kinetic properties of the compounds.

Eliminate false positives

Both SPR and ITC can be used for identification of false positives since both techniques measure direct binding of the lead to the target protein. If the target protein shows binding of a reference compound in ITC/SPR experiments but the hit does not, the compound is most probably a false positive in the HTS. In SPR, the shape of the sensorgram can be used for the identification of false positives (Fig 6).

ITC directly measures heat of interaction. The lack of saturable binding in the concentration range predicted from IC\textsubscript{50}-values is a strong indication of a false positive if target activity can be confirmed by a positive control. In the following example (Fig 7), cPLA2 catalyzes the release of arachidonic acid from membrane phospholipids to initiate the production of proinflammatory mediators such as prostaglandins. Specific inhibitors have been shown to have potent anti-inflammatory properties. However, compounds that perturb the membrane surface can be false positives in the primary assay.

The experimental design was based of the titration of candidate compound in the presence of membrane while measuring binding in the presence and absence of irreversible inhibitors. ITC showed no binding of compound 3 (Table 1), yet the primary assay indicated a 9 µM affinity. Compound 3 was nonspecifically bound to the membrane, and was thus a false positive.
Fig 7. All compounds were identified as positive in a Glu assay, however, ITC results demonstrated lack of binding of compound 3, a strong indication of a false positive. Data courtesy of Shionogi.

Table 1. Thermodynamic parameters determined for compounds identified as positive in a Glu assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>N</th>
<th>$K_D$ (µM)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$ (cal/mol/K)</th>
<th>$\Delta G$ (kcal/mol)</th>
<th>Glu IC$_{50}$ µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Compound 4</td>
<td>1</td>
<td>0.38</td>
<td>-8</td>
<td>2.7</td>
<td>-8.9</td>
<td>0.35</td>
</tr>
<tr>
<td>Pyrrolidine-1</td>
<td>1</td>
<td>0.29</td>
<td>-22</td>
<td>-44</td>
<td>-9.2</td>
<td>0.52</td>
</tr>
<tr>
<td>Pyrrophenone</td>
<td>1</td>
<td>0.15</td>
<td>-14</td>
<td>-16</td>
<td>-9.4</td>
<td>0.13</td>
</tr>
</tbody>
</table>

1Glu assay indicates a 9 µM affinity
**Lead selection and optimization**

ITC is a universally applicable method that provides affinity data as well as mechanistic information about specific noncovalent forces that might be involved in the binding between a lead compound and the target molecule. By direct measurement of heat of interactions, ITC provides insights into total binding energy (ΔG) which is directly related to the affinity (K_d). “Lock and key” forces, such as hydrogen bonds and van der Waals interactions, correlate to enthalpy (ΔH), and can be distinguished from pure hydrophobic interactions correlating to ΔS (3).

Figure 8 illustrates three different compounds, A, B, and C, which bind to a target with identical affinities, giving identical ΔG values.

![Diagram of three compounds](image)

**Fig 8.** Compound A binds to the target with good hydrogen bonding but with unfavorable conformation changes. The binding of Compound B is dominated by hydrophobic interactions, while the target binding to Compound C is favorable both with respect to hydrogen bonding and hydrophobic interaction.

Based on affinity data, the compounds appear to be identical binders. Variations in ΔH and ΔS however reveal that the binding mechanisms are fundamentally different. The favorable binding enthalpy of Compound A indicates that the interaction is driven by hydrogen bonding and van der Waals forces. The binding has unfavorable entropy, suggesting conformational effects. Compound B has a binding affinity based on hydrophobic interaction as indicated by the large favorable ΔS, in combination with unfavorable ΔH. This may be due to dehydration of the surface and a reduction in the overall number of polar interactions. Compound C has a binding affinity based on both favorable hydrogen bonding and van der Waals and hydrophobic interactions.

One strategy to maximize compound binding selectivity is to pick the compound that displays the most favorable ΔH (Fig 9). A hydroxyl group (red arrow) was introduced on the compound in Figure 8A to allow formation of a hydrogen bond, as predicted from the x-ray structure. The compound in Figure 8B was better than the compound in Figure 8A with respect to enthalpy, but would have been missed if the optimization procedure had been guided by affinity data alone. In Figure 8C, the stereochemistry of the hydroxyl substituent was modified to give a highly enthalpy-driven interaction without paying the strong entropy penalty of breaking the internal hydrogen bond. This is an elegant example of how thermodynamically-resolved affinity can be used for understanding and steering lead optimization.

![Diagram of three compounds with modifications](image)

**Fig 9.** Optimization of a lead for plasmepsin II using enthalpic energy for guidance. Data courtesy of Ernesto Freire, Johns Hopkins University.
Orthogonal approach for confident candidate selection

Traditional SAR studies use affinity ($K_D$) or $IC_{50}$, which are often related to $ΔG$ in thermodynamic terms (4) as indicators of biological activity. Often, lead optimization is mainly performed by filling lipophilic pockets in the target by adding lipophilic moieties to the structure—an entropy-driven optimization. A better response for optimization might be residence time (5) and/or enthalpic efficiency, since they better reflect the interaction between ligand and target. Typically, ligand-target complexes that form hydrogen and van der Waals bonds are enthalpically beneficial. An interaction with long residence time has higher efficacy and long dosing interval (6).

On/off rate plots from SPR analyses (Fig 10A) provide an informative way to demonstrate the resolved scale of affinities and how these affinities can be distributed over a range of on- and off-rates (7). For example, the compounds in the gray area have an affinity around 1 µM although they have very different on- and off-rates. These variations impact the residence time of a small molecule with a target, and as such, the pharmacokinetics and pharmacodynamics. Compounds with rapid on- and off-rates and rapid clearance will require frequent administration whereas compounds with slower off-rates are more likely to require less frequent dosing. A compound with a slow off-rate is also protected from enzymatic degradation by “hiding” in the binding site of the target.

ITC data can be displayed in an analogous fashion (Fig 10B). The diagonal lines in the plot represent affinities whereas the values on the x- and y-axes show the contributions from enthalpy and entropy to binding and therefore give an insight into the mechanism of binding.

With this type of resolved binding data, SARs will be much easier to follow and use for scaffold selection and lead optimization. In addition, compounds with the best mix of desired thermodynamic and kinetic parameters are readily identified (Fig 11). In most cases, an optimal compound would have a strongly enthalpic-driven interaction and slow off-rates, indicating a long residence time in the binding pocket of the target protein.

![Fig 10. By combining (A) kinetic data from SPR and (B) thermodynamic data from ITC, SARs can be resolved and the right compounds can be selected for optimization. Residence time (off-rate) and enthalpic efficiency might be better indicators of therapeutic activity than affinity only. The gray area show the large variation in different entropy/enthalpy contributions (H/S compensation) among the µM affinity binders.](image)

- Furosemide
- Ethoxzolamide

A) Kinetics

B) Thermodynamics
Efficient determination of binding to unwanted proteins

Minimized binding of drugs to unwanted proteins is as important in drug design as optimization of ligand-target interaction. Serum proteins like human serum albumin (HSA) and α₁-acid glycoprotein (AGP) are known to bind organic molecules promiscuously. These serum proteins circulate in relatively high concentrations, and therefore, already high µM affinities to plasma proteins might have a highly negative impact on drug efficacy.

ITC was utilized to measure binding affinity and binding enthalpy of different protease inhibitors to AGP and HSA (Fig 12).

SPR is an excellent tool to measure the binding to HSA and AGP simultaneously. Figure 13 shows the ranking of a number of carbonic anhydrase (CAI), kinase, and thrombin inhibitors binding to these two plasma proteins. Several of the compounds in the lead series showed unfavorable, strong binding to HSA and AGP.
**Conclusions**

SPR and ITC are techniques which can be used throughout the entire research and preclinical drug discovery process, from verification of target protein quality with DSC to establishing the mechanism of action with ITC. Secondary screening of hits from HTS and primary screening of fragment and directed libraries for elimination of false positives and for identification of true 1:1 binders are also an important application areas. SAR and QSAR studies using residence time and enthalpy are important lead optimization criteria that help to understand the efficacy of the drug and to make correct interpretation of pharmacokinetic and pharmacodynamic properties of lead compounds (5,6,8). We believe that these biophysical methods are still under-utilized and that increase in the use of DSC, ITC and SPR will improve the efficacy of the drug discovery process.

**Fig 12.** Calorimetric titrations of (A) AGP with sagunavir, (K<sub>D</sub>, 4 µM) and (B) ritonavir, (K<sub>D</sub>, 0.8 µM). These high affinities indicate that the compounds are strongly bound to plasma.

**Fig 13.** SPR-based ranking of binding to HSA and AGP for a set of inhibitors to carbonic anhydrase (C), kinase (K), and thrombin (T). Several of the lead compounds (T9, K8, K7, and C8) show very strong binding to HSA and AGP. The compounds close to 0/0 show a very weak binding to HSA and AGP.

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**References**

Overview of technologies

**Binding strength and kinetics with Biacore systems**

Biacore systems monitor molecular interactions in real-time using surface plasmon resonance (SPR). Without the need for labels, Biacore assays provide information on affinity, kinetics, and specificity of molecular interactions. The active concentration of biomolecules can also be determined.

One of the interacting molecules is immobilized onto a sensor surface, while the other molecule flows over the sensor surface in solution. Any interaction between the two is detected in real-time via changes in mass concentration close to the sensor surface and binding data is presented in a sensorgram where SPR responses in resonance units (RU) are plotted versus time.

The formation and dissociation of complexes are followed during the course of an interaction, with the binding kinetics ($k_a$, $k_d$) revealed by the shape of the binding curve.

More information can be found at [www.gelifesciences.com/biacore](http://www.gelifesciences.com/biacore)

**Binding strength and energetics with MicroCal ITC systems**

Isothermal titration calorimetry (ITC) determines many binding parameters ($n$, $K_D$, $\Delta H$, and $\Delta S$) in a single experiment without the need for labels.

A solution of one biomolecule is titrated with a solution of its binding partner and the heat released upon interaction ($\Delta H$) is monitored over time. Each peak represents a heat change associated with the injection of a small volume of sample. As successive amounts of sample are titrated into the ITC cell, the quantity of heat absorbed or released is in direct proportion to the amount of binding. At saturation, the heat signal diminishes until only heats of dilution are observed.

The binding curve, based on heat plotted against the ratio of ligand and binding partner in the cell, is analyzed to determine $K_D$, $n$, and $\Delta H$.

More information can be found at [www.gelifesciences.com/microcal](http://www.gelifesciences.com/microcal)

**Protein stability with MicroCal DSC systems**

In differential scanning calorimetry (DSC), as a biomolecule is heated at a constant rate, the heat change associated with thermal denaturation can be determined for proteins in their native state.

The measured transition midpoint ($T_m$) provides a quick and easy indication of stability, which can be used throughout the course of biotherapeutic development and formulation. Increases or decreases in thermal stability is observed as $T_m$ shifts. A higher $T_m$ reflects higher thermal stability, which correlates well with long-term stability.

More information can be found at [www.gelifesciences.com/microcal](http://www.gelifesciences.com/microcal)