Resolving bottlenecks in fragment-based lead discovery through novel SPR methodology

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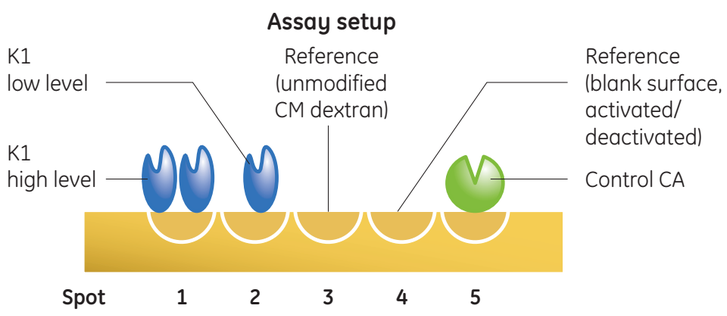
Summary

Surface plasmon resonance (SPR) biosensors can be used to screen and affinity-rank thousands of fragments consuming only microgram amounts of protein for a complete screen. We have developed new innovative methodology that supports early identification and elimination of super-stoichiometric sticky binders. Specificity of fragment hits are further confirmed using competition experiments. New strategies are used, allowing the estimation of millimolar affinities from typical fragment concentration series. Real life examples are shown using these novel SPR-based screening and affinity evaluation tools to improve selection of fragments appropriate for subsequent soaking and co-crystallization studies. For a tyrosine kinase, the binding mode of 85% of the selected fragments could be identified from x-ray electron densities, indicating that the use of SPR in this way could significantly improve the success rates of subsequent co-crystallizations.

Methods

Target proteins and assay setup

A kinase domain of a tyrosine kinase - K1 (MW 32 kDa) was immobilized at low and high levels on a CM5-sensorchip. Carbonic anhydrase was used as a control protein.

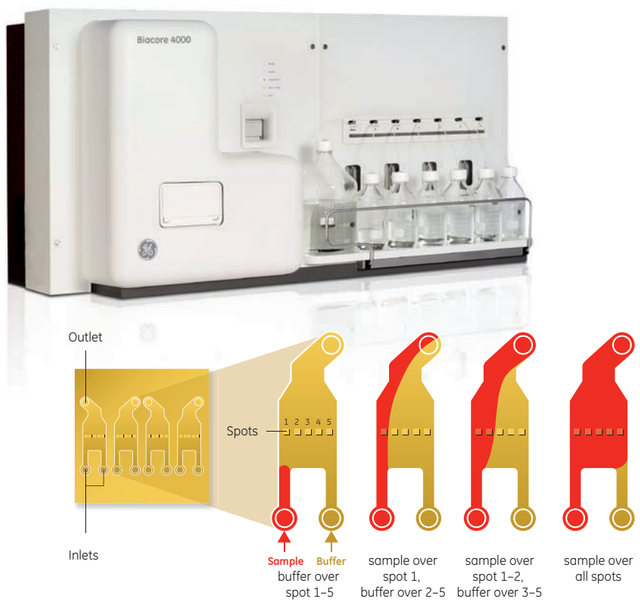


Fragment library

Unbiased, in-house library of 1920 fragments selected with a special focus on chemical diversity and solubility. The MW was between 100 and 260 Da (average 220 Da).

Biacore™ 4000

- Measure binding of 4 fragments to 4 target proteins simultaneously
- Screen 1400 fragments binding to 4 proteins in 24 h using just 20 µg of each protein
- Affinity ranking of > 200 fragments in 24 h



Four parallel flow cells with 5 spots each. Different flow rates through the 2 inlets addresses the sample over different parts of the flow cell.

Concept overview

Clean Screen
- Rapid screen of entire library against each target
- Goal: Removal of fragments that could potentially disturb subsequent assays

Binding Level Screen
- Single concentration screen
- Goal: Rapid prioritization of fragments

Affinity Screen
- Steady state analysis based on concentration series
- Goal: Affinity ranking and verification

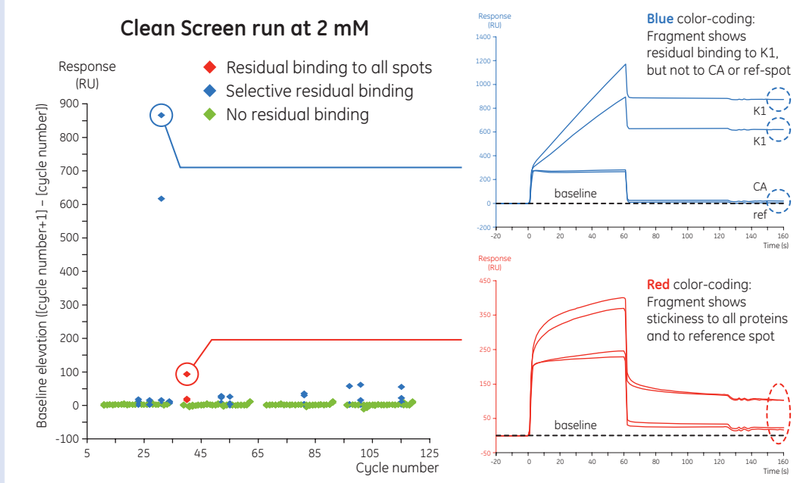
Competition assay
- Use site-specific inhibitor
- Goal: Validation and binding site mapping

Fragment Screening Strategy

Get rid of sticky compounds!

1. Clean Screen

- Screen of entire library against all targets and dextran reference
- Goal: Removal of trouble-making sticky samples



Of 1920 fragments screened, 12 (0.6%) were removed. These residual binders disturb assay performance if not eliminated from the following screens.

Get rid of non-binders!

2. Binding Level Screen

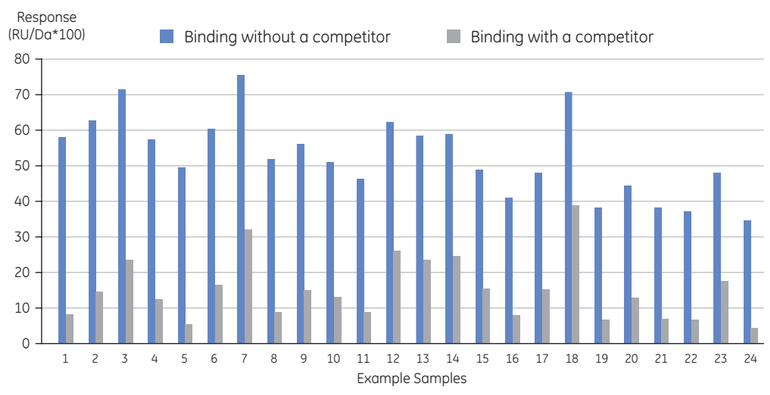
- Single concentration screen against target and reference
- Goal: Rapid prioritization of fragments in the library by automatic color labeling with binding behavior markers

No binding behavior marker indicates that a fragment has a "pure" desired interaction with the target protein.

Identify binding sites!

3. Competition assay

- Use an inhibitor in samples (and buffer)
- Goal: Fragment binding in the presence and absence of an active site inhibitor identifies fragments binding to a designed site



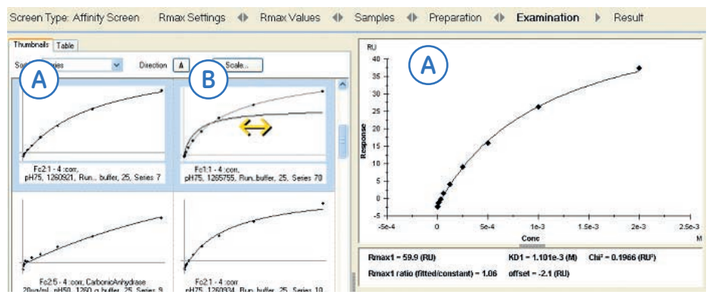
Binding inhibited by competitor shows that the binding is selective!

Measure mM affinities!

4. Affinity Screen

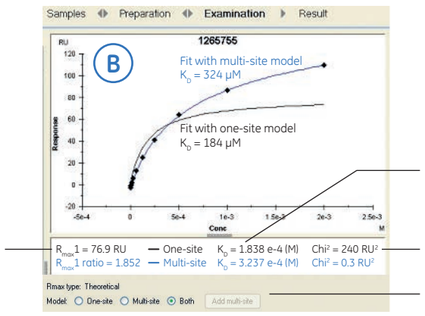
- Steady state analysis based on concentration series and predetermined R_{max} estimated with a R_{max} control. Multi-site model is used to minimize problems with secondary interactions.
- Goal: Estimate affinity using a new method which stabilizes the estimation of K_D of low affinity interactions (mM) at non-saturating concentrations and in the presence of secondary interactions.

Thumbnail overview of affinity fitting results



The multi-site model (**B**) facilitates determination of K_D of the "high" affinity site associated with predetermined R_{max} in spite of the presence of secondary interactions.

In the absence of secondary interactions, the experimental data fits well to a one-site 1:1 model when analyzed with predetermined R_{max}.



Predetermined R_{max} estimated with positive control and kept constant during fitting.

Fitting results and statistics for both models can be compared.

Statistics

User ultimately decides which model is used.

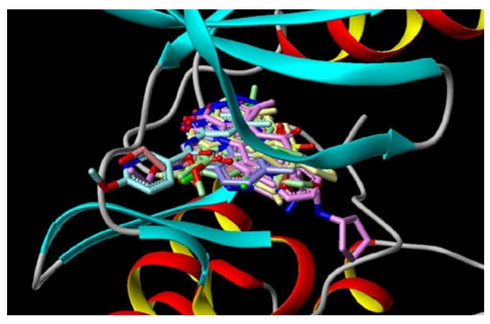
Software suggests that a multi-site model ⟷ is preferable when analyzing with predetermined R_{max} and displays an overlay plot with both models. Secondary interaction handled by the model.

High quality fragments
- Compounds without binding behavior markers
- Compounds approaching saturation of the surfaces without exceeding a 1:1 stoichiometry
- Compound for which binding was blocked by the active site-specific inhibitor in the competition experiments

Binding mode asap!

5. X-ray

- Soaking and co-crystallization experiments performed only with **high quality fragments**
- Goal: Get a binding mode of all fragments in the binding site



Overlay of 41 fragment/kinase co-structures of various chemical subtypes including unusual pharmacophores.

105 high quality fragments for K1 were identified (K_D between 13 µM and > 3 mM) and analyzed also with **Microcal™ ITC**. 80% were confirmed as binders. 48 were selected for crystallization based on chemical diversity, ligand efficiency, and affinity. For 41 fragments, X-ray structures were solved (K_D 43 µM to > 3 mM, resolution 2.0 to 2.9 Å). **X-Ray success rate was > 85%.**

Conclusions

- **Biacore systems have the sensitivity and throughput necessary to screen medium-sized libraries (1000–10 000 compounds) with low molecular weight and low affinity fragments**
- **The fragment screening process is supported by Biacore 4000 software which comes with a comprehensive toolkit, making data evaluation much easier, and leading to more confident conclusions**
- **Screening at a fragment concentration of 2 mM and choosing a competition setup allowed quick and reliable identification of millimolar binders, and resulted in a success rate of > 85% in the following crystallization trials**

imagination at work

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