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# Benefits and Drawbacks of Binding Kinetic Methodologies

# Binding Kinetic Methodologies: Choosing The Best Tool For The Job

All drugs display their efficacy and toxicity by binding to intended and non-intended targets with various levels of occupancy and duration. The ability to accurately determine **kinetic** rate constants enables compound differentiation otherwise indistinguishable by binding **affinity**. Moreover, for *in vivo* experiments with inconclusive PK–PD relationships, knowledge of drug residence time on the target of interest may be extremely helpful to explain observed data.

Several methods have been developed to determine kinetic on- and off-rates for small molecules. Factors to be considered when selecting the most appropriate method include:

**Kinetic resolution**
How to extract as much useful kinetic information as possible.

**Number of compounds and targets of interest**
Determine cost implications of assay set up and ongoing analysis.

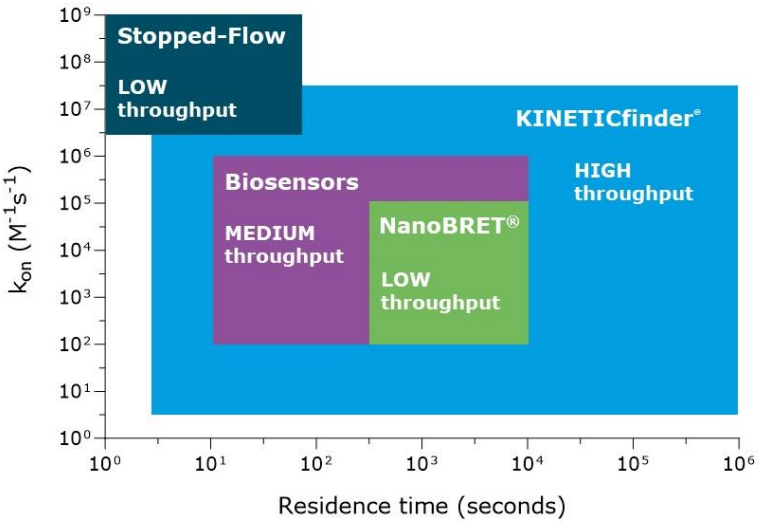
**Assay reliability**
To make decisions with confidence.

**Turnaround times**
Fit with project timelines e.g. Med Chem iteration.

**Biological relevance**
To better predict in vivo effects.

Understanding the various benefits and limitations of each methodology in terms of relevant targets, sensitivity, throughput, associated costs, turnaround and kinetic resolution is key to making the most informed assay choice. These methods can be broadly divided into four classes: Stopped-flow, Biosensor-based, Labeled and Jump Dilution.

## Kinetic Resolution & Throughput Comparison at a Glance



# Summary of Pro's and Con's of Binding Kinetic Methods

| | | |
|---|---|---|
| **Stopped-Flow** | • Useful for very fast kinetics.<br>• In solution. | • Low kinetic resolution and throughput.<br>• Tedious sample handling.<br>• Complex data analysis.<br>• Nonspecific optical signals can interfere.<br>• High protein demand. |
| **SPR** | • Label free.<br>• Versatile technique.<br>• Allows the analysis of fragments. | • Low to medium kinetic resolution and throughput.<br>• Only suitable for soluble targets.<br>• Complications due to target immobilization on the chip.<br>• Data can be hard to interpret due to heterogeneity.<br>• Mass transport and nonspecific binding limitations.<br>• Re-runs are often needed.<br>• Requires highly stable proteins. |
| **NanoBRET®** | • Expected to be better predictors for the in vivo situation.<br>• Suitable for membranes and cells.<br>• In solution. | • Low kinetic resolution and throughput.<br>• The luciferase tag may affect target localization.<br>• Target overexpression can contribute to altered responses.<br>• Low BRET signal stability (≤2 hours).<br>• Data can be difficult to interpret.<br>• Wrong estimation of target occupancy impacts off-rates.<br>• Cannot discern poorly permeable from slow compounds. |
| **Jump dilution** | • Useful for very slow kinetics.<br>• Suitable for soluble targets, multiprotein complexes, membranes, cells or tissues.<br>• In solution. | • Low kinetic resolution and throughput.<br>• Kinetic information restricted to $k_{off}$ values.<br>• Time-consuming and cost-intensive method.<br>• Prone to misleading results.<br>• Rebinding can lead to erroneous estimation of $k_{off}$ values.<br>• Requires highly stable proteins. |
| **KINETICfinder®** | • High kinetic resolution and throughput.<br>• Suitable for soluble targets, multiprotein complexes, membranes or cells.<br>• In solution.<br>• Broad dynamic range.<br>• Allows the analysis of active and inactive proteins.<br>• Allows the analysis of fragments.<br>• Useful with tight binders.<br>• Protein stability ⩾ 10 hours.<br>• Can be used at 37ºC.<br>• Can measure multiple compounds vs multiple targets on the same plate. | • Requires probe and target labeling.<br>• Need of prior knowledge about the drug mechanism of action.<br>• Long-wavelength fluorescent compounds may interfere. |

# Detailed Comparison of Binding Kinetic Methods

## Stopped-flow methods

Stopped-flow instruments consist of two or more loading syringes containing separate reactants and a stop syringe that rapidly stops the flow of the reactants in a detection chamber. These devices enable rapid mixing of reactants and continuous measurement by different detection methods[1, 2].

**Advantages**

- Used to monitor transient (from millisecond to seconds) binding and catalytic events.
- In solution.

**Disadvantages**

- Very low throughput.
  - Tedious sample handling, complex and therefore slow data analysis.
- Large sample consumption.
- High concentrations of reagents (µM).
- Limited dynamic range.
  - $k_{on}$ is well determined while $k_{off}$ may be associated with significant error.
- Interactions displaying nonspecific optical signals can interfere with detection.

## Biosensor-based methods

### Surface plasmon resonance (SPR)

Spectroscopic technique that provides label-free quantification of target-compound affinities and kinetics. The target is immobilized to a sensor chip and compounds are then run over the sensor surface. As the compounds bind to the target, it induces a change in the refractive index of the chip[1-7].

**Advantages**

- Can measure interactions in real-time without the use of labels.
- Versatile technique – a workhorse technology with use across a range of targets.
- Allows the analysis of fragments.

**Disadvantages**

- Membrane targets cause difficulties in terms of reproducibility and robustness arising from stability and orientation issues during immobilization into lipids.
- Immobilization of the target.
  - The target may become inactive or show low activity when coupled to the sensor surface.
  - The target orientation upon immobilization may sterically hinder compound binding.
  - The use of different chips when building a kinetic dataset is a source of variability.
- Mass transport limitation.
  - If the supply of compound is not sufficiently fast compared with the binding step, the observed surface-binding kinetics reflects the characteristics of the mass transport step.
  - For fast-on compounds or with high density target immobilization, mass transport limitation may hinder the accurate determination of affinity, on- and off- rate constants.
- Heterogeneity in the target, compound or in surface binding sites tends to complicate sensorgrams and reduce data reliability.

  - Target oligomerization or aggregation produce sensorgrams that are difficult to interpret.
  - Low-affinity sites resulting from partial target degradation may lead to data artifacts.
- The presence of nonspecific surface sites influences sensitivity and mass transfer.
- Signal is highly sensitive to solvent and temperature variation, affecting the reproducibility of kinetic constants.
- Low to medium throughput for the determination of kinetic data.
  - Modern devices provide kinetic resolution for up to 12 compounds at a time in 5 hours.
  - The concentrations of compounds and assay timeframe should be judiciously chosen before the run. This can be difficult without prior knowledge of the binding kinetics.
  - Fast regeneration of sensors fails with slowly dissociating compounds.
- Limited dynamic range.

# Labeled methods

## KINETICfinder®

KINETICfinder® is a highly tuned TR-FRET kinetic assay based on the binding and displacement of an active-site directed fluorescent probe. Real-time binding of the fluorescent probe is detected using a labeled anti-tag antibody, which binds to the target of interest. Binding of the fluorescent probe and labeled antibody to the target increases the TR-FRET signal, whereas displacement of the fluorescent probe with a compound decreases the TR-FRET signal[2].

**Advantages**

- Does not require target immobilization, facilitating more physiologically relevant conditions.
  - Suitable for soluble targets, multiprotein complexes, membranes or cells.
  - Supports the analysis of active and inactive proteins.
- Exceptional robustness and reproducibility.
- Very broad dynamic range for kinetic and $K_d$ measurements: >8 log.
  - The absence of preincubation steps allows $K_d$, $k_{on}$, $k_{off}$ and residence time to be measured in the same assay and a more reliable analysis of fast binders.
  - A superior target stability (≥10 h) enables the evaluation of extremely slow binders.
  - A highly sensitive method that precisely characterizes tight-binding compounds.
  - Allows the analysis of fragments.
- High throughput.
  - $K_d$, $k_{on}$, $k_{off}$ and residence time of up to 80 compound per target in one 384-plate.
  - Multiple compounds can be assayed against multiple targets in the same plate.
- Fast and cost-effective method that allows:
  - Parallel SAR and SKR to guide the selection and optimization of drug leads.
  - Modulation of the therapeutic window by fine tuning on- and off-target kinetics.

**Disadvantages**

- The requirement for a fluorescent probe and labeled target.
- Need of prior knowledge about the drug mechanism of action.
- Long-wavelength fluorescent compounds may interfere with the assay redout.

# NanoBRET®

NanoBRET® utilizes bioluminescence resonance energy transfer (BRET) in living cells by molecular proximity of an active-site directed fluorescent probe to a luciferase-fused target. Binding of the fluorescent probe to the bioluminescent target increases the BRET signal, whereas displacement of the probe with a compound decreases the BRET signal. Two different approaches can be used to determine the kinetic constants: a kinetic intra-cellular or jump dilution method[2, 8, 9].

**Advantages**

- Can yield more physiologically relevant information (receptor internalization, microenvironment, compartmentation, redox and cellular amplification of the response).
- Suitable for membranes and cells.
- In solution.

**Disadvantages**

- Requires a fluorescent probe and a bioluminescent fusion target for target labeling.
  - Addition of the luciferase tag may affect the expected target localization.
  - The fluorescent probes should be cell permeable and have rapid association and dissociation rates for the target.
  - The binding kinetics of the fluorescent probe will determine the fastest compound rates that can be monitored.
- The kinetic intra-cellular method measures forward and reverse rates of intracellular compound binding.
  - These rates are influenced both by permeability and the association and dissociation rates of the compound.
  - Therefore, this method cannot distinguish between poorly permeable or slowly dissociating and associating compounds.
- Low BRET signal stability (up to 2 hours) may hamper the accurate characterization of slow-dissociating compounds.
- Low sensitivity compared to TR-FRET methods.
- Impact of target concentration:
  - Target overexpression typically yields cellular levels (~10 nM) that are markedly different from endogenous expression (~30 fM). Disruption to the natural target stoichiometry can contribute to expression artifacts such as aggregation, mislocalization, promiscuous interactions and altered functional responses.
  - Moreover, in pharmacological assays it is assumed that the target concentration is much lower than the test compound concentration and therefore only a small proportion of the compound is bound to the target. For assay systems in which the target is highly expressed this can be a faulty assumption when compound affinity reaches the low nanomolar range. Under such conditions (referred to as tight-binding behavior), affinity values and kinetic constants can be seriously underestimated.
- The complexity of the parameters influencing cellular response can cause difficulties in interpretation of the data and erroneous estimation of target occupancy over time can have a negative influence on conclusions about off-rates.
- Need of prior knowledge about the drug mechanism of action (competitive vs allosteric).
- Low throughput.
- Limited dynamic range.

# Jump dilution or washout methods

The jump dilution method is commonly used to evaluate the reversibility of inhibition and to quantify the dissociation rate of the target-compound complex. In a jump dilution assay, the target is preincubated at 100-fold the concentration required for assay with a saturating concentration of compound ($10xIC_{50}$) to achieve 90% occupancy. Once sufficient time has elapsed to achieve equilibrium, the sample is diluted by 100-fold to give 9% occupancy. Then, the target occupancy is measured as a function of time[1-3, 10, 11].

**Advantages**

- Used to monitor extremely slow dissociating compounds.
- Does not require target immobilization, facilitating more physiologically relevant conditions.
- Suitable for biochemical activity assays, disease-relevant cellular assays or *in vivo* functional assays.
- In solution.

**Disadvantages**

- Compounds with short residence time can display much longer residence time when:
  - $IC_{50}$ has been overestimated: The compound is dosed at a higher concentration in the preincubation step and consequently, even in presence of an excess of unlabeled competitor, the compound rebinds with the target during the washout step.
  - Washout is incomplete: Some compounds are not easily washed out and may accumulate at high concentrations. Hence, remaining compound can re-associate with the target.
- Compounds with long residence time can display much shorter residence time when dealing with high-affinity inhibitors that display tight-binding behavior:
  - The concentration of compound needed to achieve 90% target occupancy is well below target concentration. Consequently, target occupancy recovery of slow-dissociating, tight-binding compounds are comparable to rapidly reversible inhibitors.
- Reversible but slowly dissociating compounds may appear to be irreversible:
  - When initial $IC_{50}$ determinations indicate tight-binding behavior, preincubations should be performed at very high compound concentrations to ensure significant target occupancy during the preincubation step. However, increased compound concentration during preincubation results in reduced recovery of target occupancy after dilution.
- Limited dynamic range.
  - Time-dependent loss of target stability due to spontaneous target denaturation, degradation or unfolding may limit the accurate characterization of slow-dissociating compounds.
  - Not suitable for compounds with a residence time < 1 hour: Compounds with residence times less than one-sixth the time window of analysis, display occupancy recoveries lower than expected for a rapid dissociating compound.
- Low throughput.
  - Prior to determining $k_{off}$, different assays must be run to identify the optimal enzyme concentration, preincubation time and the $IC_{50}$ values of the inhibitors for which off-rates are to be measured. This can be difficult without prior knowledge of the binding kinetics.
  - If tight binding is suspected, then $IC_{50}$ and $k_{off}$ values should be determined at more than one enzyme concentration.
  - Tedious sample handling.

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