# Binding kinetics: high throughput assay for kinase inhibitors

Krumm A¹, Georgi V², Schiele F², Fernández-Montalván A²

¹ BMG LABTECH GmbH, ² Bayer AG, Drug Discovery, Pharmaceuticals, Berlin, Germany

- Microplate-based TR-FRET assay determines target binding kinetic rates $k_{on}$ and $k_{off}$ for kinase inhibitors
- Speed of the PHERAstar® offers high temporal resolution and high throughput kinetic characterization
- Screening of interaction between multiple inhibitors and kinases relates $k_{on}$ and clinical success

## Introduction

Drug development is a lengthy and costly process with high attrition rates. More than half of the drug candidates going into phase 2 clinical trials fail mainly due to lack of effectiveness¹. A feature associated to the efficacy and safety of drug candidates is how fast they bind ($k_{on}$) and dissociate ($k_{off}$) from their targets, and accordingly for how long they can occupy them (residence time). Ideally these kinetic parameters are determined in the preclinical stages, creating a need for high-throughput methods to profile hundreds and thousands of compounds. Moreover, comprehensive retrospective profiling of existing molecules from different clinical development stages can provide insights into the impact of target binding kinetics on the pharmacological profiles of drugs.

Here, we present the determination of $k_{on}$ and $k_{off}$ values in a PHERAstar FS microtiter plate reader using time-resolved FRET (TR-FRET) for a panel of 40 kinase drug targets in cancer therapies against 270 inhibitors.

## Assay Principle

The kinetic probe competition assay (kPCA) detects the time resolved energy transfer (TR-FRET) from a lanthanide-based donor fluorophore linked to the target molecule via affinity reagents, to an acceptor dye conjugated tracer known to bind to the target of interest (and consequently found within Foerster distance).

In order to determine the kinetics of binding, target and fluorescent tracer pairs are first characterized by monitoring the TR-FRET signals during binding and dissociation of both interaction partners.

Once association and dissociation rates of the tracer are known, the same parameters can be determined for unlabelled compounds in a competitive setup where tracer and competitor molecules are simultaneously exposed to the target. In this context, binding of the competitor molecule alters the signal curve of interaction between tracer and target in a dose-dependent fashion.

Kinetics and affinity parameters of tracer and competitors can be derived by fitting the resulting signal to appropriate mathematical models² (Fig. 1).

## Materials & Methods

- Kinase Inhibitors (SelleckChem)
- Alexa 647 labeled kinase tracers (ThermoScientific)
- Biotinylated kinases (Carna Biosciences)
- Streptavidin-Terbium (Cisbio)
- black 384 well small volume microplate (Greiner)
- PHERAstar® FS (BMG LABTECH)

## Experimental procedure

For a detailed description please refer to Georgi et al. (2018)⁴. Briefly, assay plates were prepared to contain 5 µl of the fluorescent tracer with different concentrations of the competitive molecule. The reaction was started and immediately monitored by adding 5 µl of terbium-labelled kinase to the wells with a PHERAstar FS (with a sample injection unit).

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Fig. 1: Principle of the kinetic probe competition assay. Competitive compound and fluorescent tracer are placed in the microplate and the reaction is started by adding the Tb-coupled kinase (left). TR-FRET signal is recorded immediately after target addition and depends on the presence of competitive kinase inhibitors (middle). The deviation of the tracer-only curve to the tracer + competitor curve provides information about the binding kinetics of the unlabeled competitor molecule (right).
Results & Discussion

In a first step the association and dissociation kinetics of a fluorescent tracer binding to a kinase e.g. ABL was analysed. TR-FRET signals increase with times, indicating increased tracer-kinase binding, whereas the signal decreases when being displaced by an excess of unlabelled competitor (Fig. 2).

Next, an unlabeled compound was analyzed using the kinetic probe competition assay. The reaction contained fluorescent tracer 178 and the indicated concentrations of staurosporine, an unlabelled ABL-binder. The reaction was started by adding Tb-labelled ABL resulting in a signal increase for the trace without competitor (Fig. 3, grey). In contrast, the signal in presence of the competitor is lower (indicated by high negative decadic off-rate logarithm). On the other hand, the graph illustrates that the association rate of preclinical compounds is barely different from that of approved drugs. All in all, these data suggest that the longer a kinase is occupied by a compound, the greater the likelihood for it to become a clinically effective drug.

Conclusion

The TR-FRET-based kinetic probe competition assay determines binding kinetics of unlabelled compounds in high throughput. The method allows linking binding parameters to clinical success, and the selection of new drug candidates for development according to their binding kinetic properties.

References

4. Georgi et al. [2018] doi: 10.1021/jacs.8b08048