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A Guide to Measuring Drug-Target Residence Times with Biochemical Assays

During drug development initiatives, analysis of drug-target residence times can improve efficacy, increase therapeutic window, and reduce the risk of premature focus on candidate compounds that are likely to have undesirable side effects. This guide provides technical background on concepts and techniques for use of Transcreener® biochemical assays to measure drug-target residence times, along with examples and case studies.

Introduction

Assays for interactions between drug molecules and their targets have classically been measured by calculating binding affinity under equilibrium conditions. In drug discovery, it had long been assumed that carefully measuring IC_{50} (half-maximal inhibitory concentration), EC_{50} (effector concentration for half-maximal response), k_d (equilibrium dissociation constant), and/or k_i (inhibition constant) would allow drug candidates to be prioritized effectively for clinical studies.

Unfortunately, 90% of small-molecule drugs have failed in the clinic due to unfavorable pharmacodynamic properties[1], suggesting that binding affinity and specificity can be less relevant than had initially been assumed. In 2006, Robert Copeland and colleagues David Pompliano and Thomas Meek published the residence time model, which accounts for the conformational dynamics of target macromolecules that affect drug binding and dissociation.[2]

The residence time model postulates that the total lifetime (i.e., residence time, τ, or tau) of the binary drug–target complex, not the binding affinity per se, dictates much of *in vivo* pharmacological activity. Drug-target residence time is calculated as the reciprocal of the dissociation constant, k_{off} and can vary from seconds to days. Longer residence times can sometimes lead to so-called durable pharmacodynamics, where residence time exceeds the pharmacokinetic half-life of the drug in systemic circulation, resulting in sustained drug activity even when the agent is cleared from the body.[3] Durable pharmacodynamics can be desirable in some instances such as anticancer drugs, and undesirable in other cases such as ion channel blockers where toxicity is a concern, or as illustrated by the antipsychotic drug haloperidol where prolonged residence time has been postulated to contribute to undesirable side effects.[4] Thus, determining residence time can provide actionable information for drug discovery.

For enzyme drug targets, the jump-dilution method is a powerful approach for measuring residence time using more physiologically relevant conditions than biophysical techniques such as surface plasmon resonance (SPR). In a jump-dilution experiment, the enzyme and inhibitor are allowed to form a complex at saturating concentrations. The complex is then diluted rapidly and the recovery of the enzyme activity is monitored continuously as the inhibitor dissociates, and k_{off} is estimated from the rate of activity recovered. A typical jump-dilution experiment involves the following steps:

1. Incubate the enzyme with a saturating concentration of inhibitor (e.g., 10 × IC_{50}).
2. Dilute the enzyme-inhibitor mixture 100-fold into a solution containing all enzyme reaction components and detection reagents.
3. Measure enzyme activity continuously to monitor the recovery of activity over time.
4. Determine k_{off} by fitting enzyme progress curves to an integrated rate equation:

$$P = V_S t + (V_0 - V_s)\frac{\left(1 - e^{-k_{off}t}\right)}{k_{off}}$$

Integrated Rate Equation. *The residence time is calculated as an inverse of k_{off}.*

Using an activity-based approach means that there is no requirement for labeled ligands, which can be cost-prohibitive. Also, immobilizing target proteins as in SPR requires time and optimization, and may not reflect binding dynamics in biological systems. Of the various techniques suitable for measuring residence time, each has inherent pros and cons (see Table 1).

Method	Description	Advantages	Disadvantages
Surface Plasmon Resonance (SPR)	Monitoring dissociation of inhibitors from immobilized protein by detecting changes in angle of reflected polarized light in flow cells; the angle changes as molecules bind and dissociate[5]	• Label-free detection • Compatible with small samples • Sensitivity	• Cost • Dedicated instrument required • Target enzyme immobilization can artificially constrain conformation • Low-throughput
Biolayer Interferometry	Quantifies in real time the interference pattern of white light reflected from a layer of immobilized protein on a biosensor tip and an internal reference layer; changes in the number of molecules bound to the biosensor tip causes an interference pattern shift[6]	• Label-free detection • Real-time • Compatible with crude samples • Doesn't require reference channel	• Lower sensitivity • Target enzyme immobilization to sensor tip can artificially constrain conformation
Resonant Acoustic Profiling	Monitoring interaction of ligand and target immobilized on a crystal by detecting modulation of the acoustic resonance of the crystal[7] (detects the sounds of bonds breaking)	• Label-free detection • Compatible with crude samples • Real-time	• Requires a variety of integrated technical controls to facilitate sensitivity, accuracy and precision
Jump-Dilution Assay	Monitoring enzyme activity over time as dilution drives inhibitor dissociation[8]	• Economical • Does not required dedicated instrumentation • Sensitivity • Does not require immobilization • HTS Compatible	• Not suitable for non-catalytic target-ligand interactions

Table 1. *Methods for determining residence time.*

With jump-dilution assays, enzyme interrogation can be conducted with native substrates at their physiological concentrations. The process does not require prior knowledge of inhibitor mechanism, binding site for the inhibitor (e.g., catalytic site or allosteric site), or whether the inhibitor competes with enzymatic substrates.[4] Moreover, because jump-dilution assays measure the dynamic interactions between an enzyme, its substrates, and inhibitors during catalysis, the technique may reveal aspects of inhibitor binding that would not be observed in simple binding assays.[4]

Residence Time Assays Provide Insight Into Drug-Target Interactions

In the static model of drug-target interactions (see Figure 1), the target macromolecule contains a binding pocket that is sterically and electronically complementary to the ligand, establishing a network of favorable interactions within the binding pocket and stabilizing the binary drug-target complex relative to free reactants.[8,9] This conventional view considers the recognition elements of the binding pocket to be held static in the most complementary arrangement with respect to ligand interactions. Hence, drug association and dissociation each occur in a single kinetic step, and the efficiency of interaction may be quantified by familiar, mathematically related parameters such as IC_{50}, k_d, etc. In reality however, this is not very common and most drug-target interactions require some conformational modulation to occur.

An alternative model considers conformational adaptation in enzyme-inhibitor interactions and accounts for induced-fit processes (see Figure 2). In jump-dilution assays, no assumptions are made about the conformational profile of enzymes, and the conformational mobility of the enzyme is not restricted in any way. Ligand-induced changes in conformation can proceed in a manner that is more likely to occur in the cell.

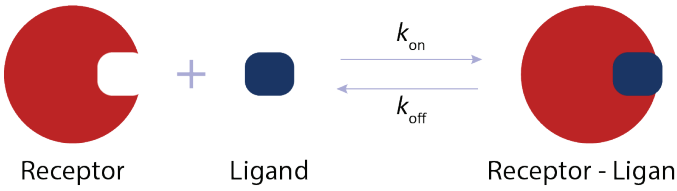


Figure 1. *Static view of drug-target interactions. Adapted from Copeland 2016.*

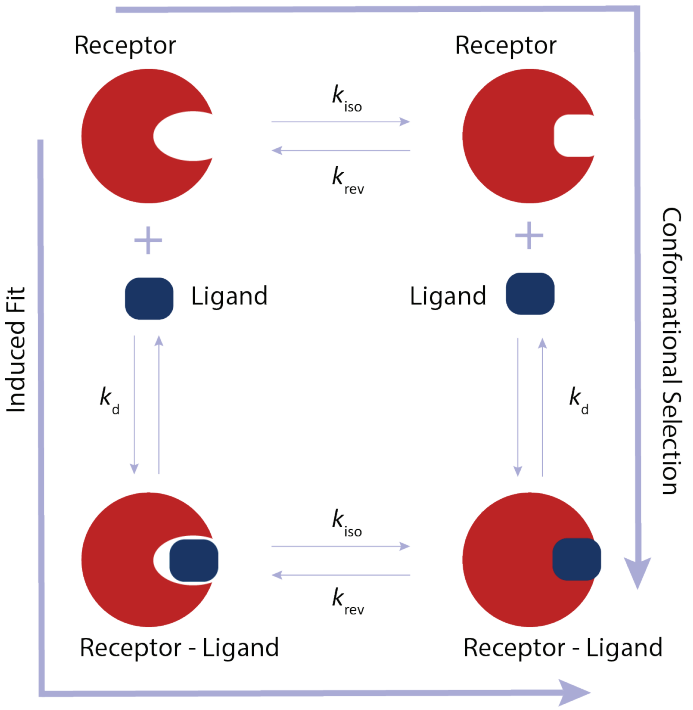


Figure 2. *The role of induced fit in conformation adaptation. Adapted from Copeland 2016.*

Transcreener Assay Basics

Transcreener is a universal high throughput screening (HTS) platform for enzymes that has been widely used in drug discovery since 2006. The platform provides a simple, homogenous method for directly detecting nucleotide products of enzyme reactions, e.g. ADP for kinases. Transcreener offers three choices of readout: fluorescence polarization (FP), time-resolved Förster resonance energy transfer (TR-FRET), and fluorescence intensity (FI). Direct immunodetection of nucleotides is unique to Transcreener (see Figure 3). The method is based on the interaction of two detection reagents: an antibody and a tracer. Displacement of the tracer by the nucleotide causes a change in its fluorescence properties, providing a way to measure enzymatic activity.

The approach is highly amenable to HTS, with reagents and instrumentation readily available for key target families like kinases, glycosyltransferases, and phosphodiesterases. Therefore, an activity-based approach such as the Transcreener jump-dilution method provides a way to incorporate residence time measurements early in lead discovery. Other universal assay approaches use coupling enzymes that require the assay to be performed in endpoint mode, which makes determination of residence times with the jump-dilution approach not viable.

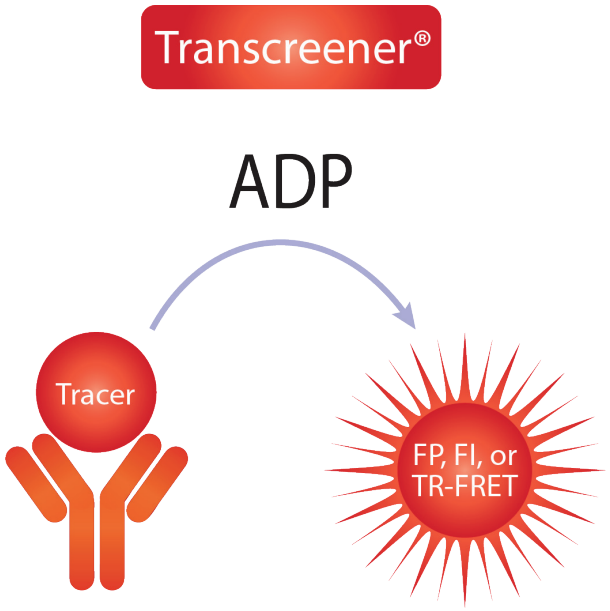


Figure 3. *Overview of the Transcreener Assay using nucleotide immunodetection and FP, FI, or TR-FRET fluorescent readouts. This example uses ADP however, GDP, UDP, AMP/GMP, and SAH assays have also been developed.*

Detection Methods

Residence time determination can be achieved with any of three different assay formats. With FP, a fluorophore is excited with linearly polarized light and measured through an emission polarizer that is placed either parallel or perpendicular to the exciting light's plane of polarization.[10] TR-FRET generates a signal via fluorescent resonance energy transfer between a donor and an acceptor molecule when they are in close proximity to one another. Dual-wavelength detection allows reduction of buffer and media interference, and the resulting signal is proportional to product formed.[11] FI involves detecting a simple fluorescent intensity output, which is readily measured by basic fluorescence readers and more complex multi-mode plate readers alike.

A head-to-head comparison of FP, FI, and TR-FRET assay formats for EGFR inhibitor drugs was performed with the Transcreener ADP^2 Kinase Assay to determine whether the orthogonal assays would produce equivalent results (see Table 2). The assays all use the same ADP antibody, but each conjugation state, as well as the tracer structure, is different for each detection readout.[4] And although the trends are similar, the absolute values for residence time do vary slightly depending on assay format.

	Compounds	FP	FI	TR-FRET
EGFR EC_{80} (nM)		2	3.4	5
IC_{50} (nM)	Afatinib	1.2 ± 1.4	1.3 ± 0.9	1.6 ± 0.7
	Lapatinib	0.36 ± 1.1	0.1 ± 1.3	0.15 ± 1.2
	Erlotinib	7.7 ± 1.5	11.5 ± 1.4	10.9 ± 0.7
	Gefitinib	7.3 ± 1.7	11.4 ± 0.7	13.3 ± 0.8
Tau (min)	Afatinib	215 ± 16	105 ± 27	365 ± 19
	Lapatinib	108 ± 8.3	96 ± 22	124 ± 11.8
	Erlotinib	30 ± 2.8	69 ± 12.7	15 ± 2.7
	Gefitinib	2 ± 5	<1	<1

Table 2. *Equivalency of Transcreener ADP^2 Kinase Assay Formats. Potencies and residence times for EGFR inhibitors were measured with the Transcreener ADP^2 Kinase Assay in FP, FI, or TR-FRET formats.*

Of the three assay formats, FP holds some distinct advantages for residence time determination. For example, studies by BellBrook Labs comparing Transcreener ADP^2 jump-dilution assays of ABL1 kinase found that FP format is the most robust and sensitive.[4] Since the FP assay detects displacement of the tracer as a decrease in polarization due to increased rotational mobility, the assay is homogenous, rapid, and in mix-and-read format. Importantly, it can be used in kinetic mode, meaning that it can be conducted with the detection reagents present during the kinase enzymatic reaction.

A requirement of the jump-dilution method is that the enzymatic activity in the reaction time course following dilution must be observable with the assay method used. For Transcreener FI and TR-FRET assays, the kinetics of the antibody–tracer binding reaction do impose a limitation on measuring very short residence times, and conversely, if residence time is very long, it may not be possible to continue the post-dilution activity assays long enough to obtain sufficient curvature.[4] The reagent and signal stability for the Transcreener ADP^2 assay using FP mode are both greater than 24 hours. Therefore, the assay imposes fewer limitations on the period of activity measurements.[12] Loss of enzyme activity due to denaturation is more likely to limit the ability to observe very slow changes in velocity than assay instability.

Feasible and Unfeasible Target Types

A common question regarding the use of Transcreener technology to determine residence time is, "Which target types are compatible with this technique?" The robust

Transcreener approach is appropriate for jump-dilution assays to determine residence time for many different target-ligand types, including:

- **Kinases and ATPases:** Transcreener ADP^2 Assay
- **Phosphodiesterases:** Transcreener AMP^2/GMP^2 Assay
- **Glycosyltransferases:** Transcreener UDP^2 Assay

There are however, certain targets for which Transcreener or AptaFluor® jump-dilution assays are not suitable for residence time determination. These include:

- **GTPases:** Use of Transcreener GDP GTPase Assays for quantitating residence time is not recommended. The inherently slow turnover of GTPases[13] renders the technique unsuitable.
- **Methyltransferases:** The EPIGEN SAH Methyltransferase assay is designed for endpoint readout, and cannot be used for determination of residence time because assay equilibration does not occur rapidly enough. For more information, see sections 4.5.1 and 4.5.2 of the EPIGEN SAH Methyltransferase Assay technical manual. Additionally, the AptaFluor SAH Methyltransferase Assay, which uses a novel split aptamer approach with a TR-FRET readout to measure enzyme activity, is also designed for endpoint readout. AptaFluor is also not recommended for determination of residence time due to long assay equilibration time during incubation (3 hours). It is critical the assay detect enzymatic activity on a near real-time basis in order to accurately determine residence time with the jump-dilution technique.

Example 1: Abl1 Kinase Inhibitors

This example provides a jump-dilution protocol for determining the dissociation rate constant k_{off} using the Transcreener ADP^2 Assay with the target kinase Abl1 and inhibitors Dasatinib, Ponatinib, Nilotinib, and Imatinib.

Materials

- Black, Non-Binding, low volume (LV) 384 well plate (Catalog #4514, Corning)
- Transcreener ADP^2 FP Assay (Catalog # 3010-1K or 3010-10K)
- Plate reader (e.g., Tecan Safire)
- Expressed, purified kinase(s) of interest
- Inhibitor(s) of interest
- Kinase buffer (e.g., 50 mM Tris (pH 7.5), 5 mM $MgCl_2$ and 0.01% Brij® 35)

Protocol

I. Determine optimal enzyme concentration and IC_{50} value.

Prior to determining k_{off}, it is important to identify the optimal enzyme concentration and the IC_{50} values of the inhibitors for which off rates are to be measured. Detailed methods to determine the optimal enzyme concentration and IC_{50} values are beyond the scope of this guide; however, an example is given below.

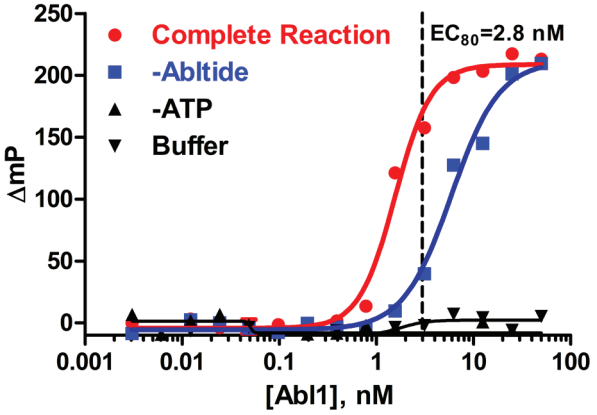
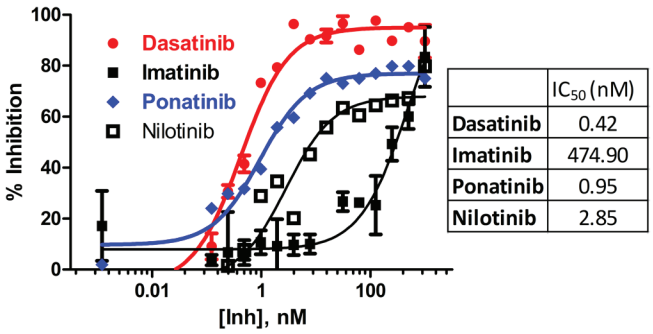


Figure 4. *Abl1 enzyme titration in the presence of 5 µM ATP and 10 µM Abltide. A concentration (EC_{80}) of 2.8 nM was determined to be optimal from this titration. The reaction was run in a kinase buffer (50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 0.01% Brij).*



	IC_{50} (nM)
Dasatinib	0.42
Imatinib	474.90
Ponatinib	0.95
Nilotinib	2.85

Figure 5. *A dose response curve for Abl1 enzyme (2.8 nM) in the presence of 5 µM ATP and 10 µM Abltide. IC_{50} values of 0.42 nM (Dasatinib), 475 nM (Imatinib), 0.95 nM (Ponatinib), and 2.8 nM (Nilotinib) were determined based on this experiment.*

II. Jump-dilution method

II-a. Preincubation protocol

1. Select an amount of kinase for the preincubation step such that, after dilution in the binding assay, the resulting concentrations of kinase will still provide a robust signal. It is recommended to use a kinase concentration equivalent to the EC_{80} value × 100.

2. In general, the inhibitor concentration chosen should be 5 to 20 times the IC_{50}. For this example ~10 x IC_{50} was used.

3. For the example described above with Abl1, reagent concentrations were 280 nM enzyme, 4.5 nM Dasatinib, 10 nM Ponatinib, 4.5 µM Imatinib, and 25 nM Nilotinib. DMSO alone controls (no inhibitor) were also run simultaneously. A control that lacks enzyme is also critical to allow calculation of maximum inhibition.

4. To permit the [EI] complex to form, incubate 10 µL of Abl1 enzyme with 10 µL of inhibitor/DMSO in 50 mM Tris (pH 7.5), 5 mM $MgCl_2$ and 0.01% Brij for 1 hour at room temperature.

II-b. Dilution and measurement

1. The dilution step should bring the concentration of inhibitor well below its IC_{50} value. Thus, the 10 x IC_{50} preincubations were diluted 100-fold: 0.2 µL of Abl/Inhibitor mixture was added to 19.8 µL of detection mixture in a low-volume 384 well plate.

2. For Abl1, 5 µM ATP and 10 µM Abltide was used in kinase buffer containing 2 nM tracer and 3.2 µg/mL of ADP^2 antibody.

3. The plate was mixed well and read kinetically every 5 minutes for 4 hours in a Tecan Safire plate reader using the fluorescent polarization mode with excitation (EXC) at 630 nm and emission (EMS) at 670 nm.

4. A standard curve under similar conditions was also run kinetically at 5 µM ATP/ADP at various percent conversions in the presence of 10 µM Abltide to enable converting the raw polarization data into product formed (µM ADP).

II-c. Controls required for data analysis

To calculate initial velocity (V_0), perform an uninhibited Abl1 reaction by running 2.8 nM enzyme in the presence of 5 µM ATP and 10 µM Abltide.

To calculate steady-state velocity (V_s), run a fully inhibited Abl1 reaction by running enzyme in the presence of 100 × IC_{50} of any potent inhibitor. In this example we used 2.8 nM Abl1, 400 nM Dasatinib in the presence of 5 µM ATP and 10 µM Abltide.

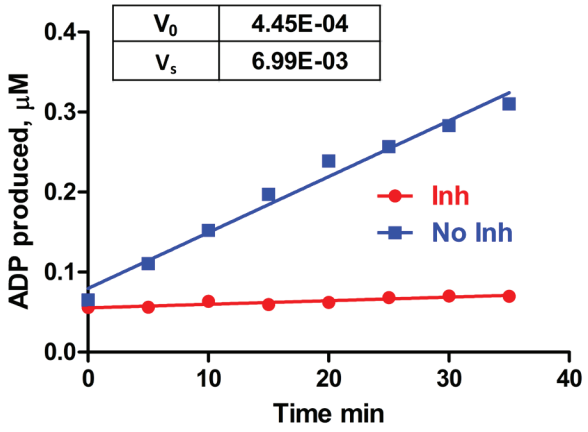


Figure 6. *Enzyme velocity V_0 was determined for fully inhibited reactions using Abl1 enzyme, 2.8 nM in the presence of 400 nM Dasatinib, 5 µM ATP and 10 µM Abltide. Enzyme velocity V_s was determined for un-inhibited reactions using Abl1 enzyme, 2.8 nM in the presence of 5 µM ATP and 10 µM Abltide.*

III-c. Data analysis

Data conversion

1. Data analysis was performed using the GraphPad Prism software package.

2. The polarization data was converted into product formed (µM ADP) using a standard curve described in the ADP^2 FP Assay Technical Manual.

3. Data that shows less than 30% consumption of ATP should only be used to ensure initial velocity conditions.

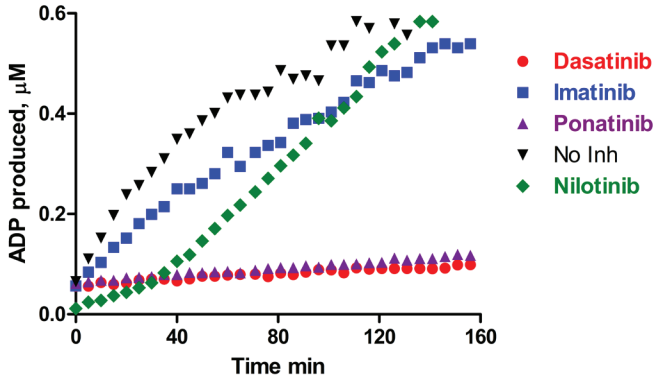
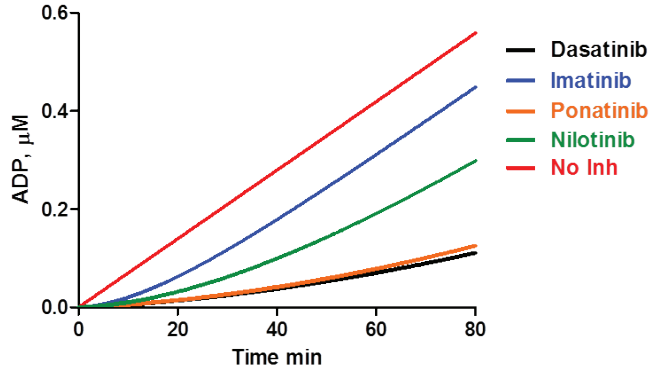


Figure 7. *Linear relationship between reaction progress times and ADP formation. The polarization values were converted into ADP (product formed) using a standard curve set up under similar conditions.*

Determining k_{off} and residence time by fitting a curve to the equation in GraphPad Prism.

Substitute the values of V_0 and V_S from Figure 6 into the integrated rate equation.

1. Obtain this equation: Y=0.006985t-0.00654/K(1-exp-K*X)
2. Open the analysis window in GraphPad Prism and select New Equation.
3. Enter the new equation.
4. Set "rules"- Define K as 1/Value of X at Y_{max}.
5. Fit the curve in Graph Pad using this equation to determine the k_{off} values.



	Koff, min-1	Tau, min
Dasatinib	0.004011	249
Imatinib	0.05884	17
Ponatinib	0.004882	205
Nilotinib	0.0201	50

Figure 8. *Residence times for Dasatinib, Imatinib, Ponatinib, and Nilotinib were determined for Abl1 in a jump-dilution experiment using the Transcreener ADP² Assay. Tau values were calculated by taking the reciprocal of k_{off}.*

Example 2: EGFR

In this example, the Transcreener ADP² Assay is used to determine residence time of inhibitors Erlotinib, Gefitinib, Lapitinib, and Afatininib with kinase target epidermal growth factor receptor (EGFR).

EGFR inhibitors preincubation protocol.

To facilitate robust signal after dilution, the amount of EGFR used for preincubation is at least 100 times the EC_{80} value. The inhibitor concentration is 10 x IC_{50}, such that the enzyme is saturated with the inhibitor. The mix was incubated for 1 hour at room temperature to ensure formation of the [EI] complex.

Enzyme for [EI]

100 x EC_{80}= 2 nM x 100 = 200 nM

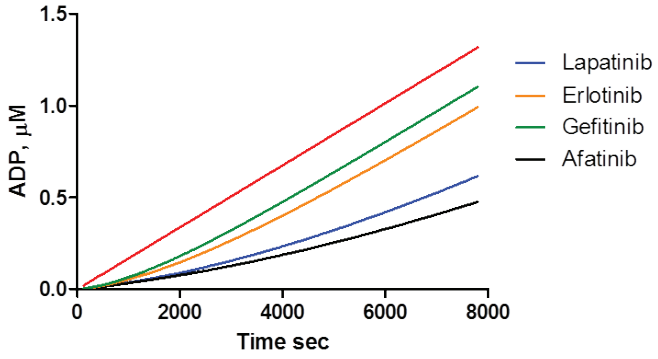
Drug for [EI]

10 x IC_{50} (Lapitinib) = 36 nM
10 x IC_{50} (Erlotinib) = 800 nM
10 x IC_{50} (Gefitinib) = 800 nM
10 x IC_{50} (Afatinib) = 120 nM

The jump dilution assays were conducted according to standard protocols as described above and in the Transcreener ADP² Assay Technical Manual. In calculating K_{off} and residence time by substituting the values of V_0 and V_s into the equation described in Example 1. A comparison to literature values can be seen in Table 3.

Kinase	Compound	Examples 1 & 2 (min)	Published Method (min)
ABL1	Dasatinib	249 ± 34	60 (SPR), 1500 (LDA)
	Ponatinib	205 ± 18	260 (SPR), 1500 (LDA)
	Imatinib	17 ± 1.7	3 (SPR), 2 (LDA)
	Nilotinib	50 ± 4.2	105 (LDA)
EGFR	Afatinib	215 ± 16	>2000 (SPR)
	Lapatinib	108 ± 8.3	354 (SPR), 300 (JD)
	Erlotinib	30 ± 2.8	5 (SPR), <10 (JD)
	Getfitinib	2 ± 5	2 (SPR), <10 (JD)

Table 3. *Residence time comparison of Examples 1 and 2 for ABL1 and EGFR compared to published values using surface plasmon resonance (SPR), ligand displacement assays (LDA), and non-Transcreener jump-dilution assays (JD).*



	Koff, min-1	Tau, min
Lapatinib	0.00925	108
Erlotinib	0.03333	30
Getfitinib	0.5	2
Afatinib	0.00465	215

Figure 9. *Residence time for inhibitors of EGFR using product formed and the integrated rate equation.*

Example 3: GALNT3 Glycosyltransferase

In this example, the residence time of two compounds of interest (referred to as Compound 1 and Compound 2) for the glycosyltransferase GALNT3 were determined by a jump-dilution assay using the Transcreener UDP^2 Glycosyltransferase Assay. The dilution of the [EI] complex was conducted such that the concentration of each inhibitor was at least 100-fold below its IC_{50} value.

The amount of inhibitor compound used for preincubation was:

Enzyme for [EI]
100 x EC_{80}= 1.5 ng/µL = 150 ng/µL

Drug for [EI]
10 x IC_{50} (Compound 1) = 50 µM
10 x IC_{50} (Compound 2)= 500 µM

A 100-fold dilution (0.2 µL of GALNT3/Inhibitor mixture into 19.8 µL of Detection Mixture) was prepared in an low volume 384 well plate. The plate was mixed well and read kinetically every 5 minutes for 4 hours in a Tecan Safire plate reader using the FP mode with EXC at 630 nm and an EMS at 670 nm. Raw data for jump-dilution experiments are shown in Figure 10.

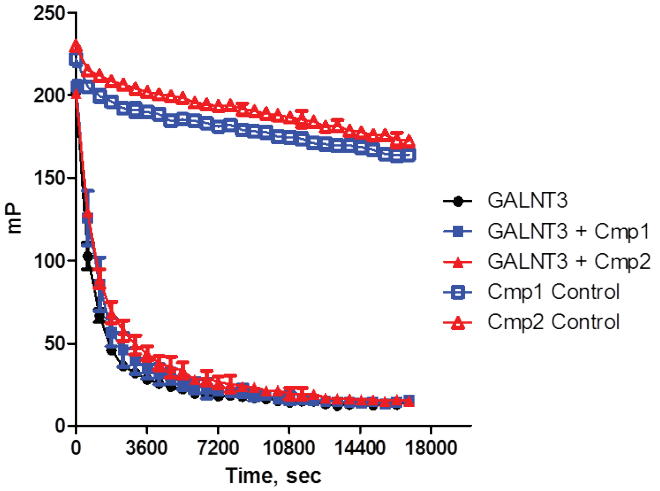


Figure 10. *Raw data from a jump dilution of GALNT3 with Compounds 1 and 2.*

Product formation over time was determined for each of the two compounds by using a UDP standard curve. Data was then transformed with GraphPad Prism to illustrate the inhibitor dissociation using raw data.

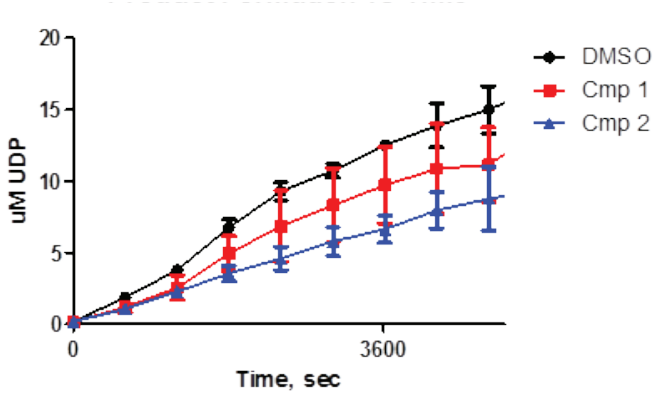
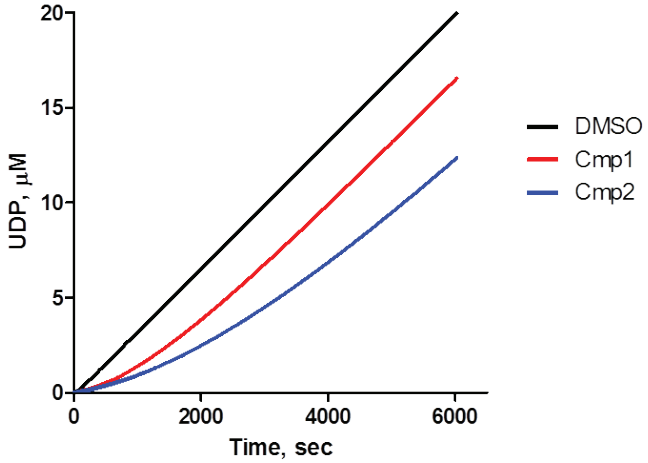


Figure 11. *Product formation versus time for Compounds 1 and 2 with the target GALNT3.*

k_{off} values were calculated as described in Examples 1 and 2. Data was then converted to determine the residence times using the product formed (UDP).

Accordingly, the residence time of Compound 1 was calculated as 22 minutes and the residence time of Compound 2 was 55 minutes.



	Compound 1	Compound 2
K	0.0007726	0.0003015
Tau, Min	21.6	55.3

Figure 12. *Residence time of Compound 1 and 2 with GALNT3 using product formed and the integrated rate equation.*

Example 4: PDE7A Phosphodiesterase

In this example, the Transcreener AMP²/GMP² Assay is used to determine residence time of inhibitors TC3.6, BRL50481, and Zardaverine on the phosphodiesterase PDE7A. This enzyme is a high-affinity cAMP-specific phosphodiesterase that is expressed in T cell lines, peripheral blood T lymphocytes, epithelial cell lines, airway and vascular smooth muscle cells, lung fibroblasts, and eosinophils. PDE7A plays a critical role in the regulation of human T cell function, and selective PDE7A inhibitors are being examined to treat immunological and inflammatory disorders. Additionally, PDE7A plays an important role in the regulation of osteoblastic differentiation: PDE7A depletion by RNAi up-regulates expression of several osteogenic genes and increases mineralization.

A 30-minute time point titration assay was conducted using Enzyme Buffer with a composition of 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, substrate concentration of 1 µM cAMP, antibody concentration of 1 µg/mL, and tracer concentration of 2 nM. The optimal concentration of PDE7A was determined to be 5 pM. Raw data and converted data expressed as product formed are shown in Figures 13 and 14, respectively.

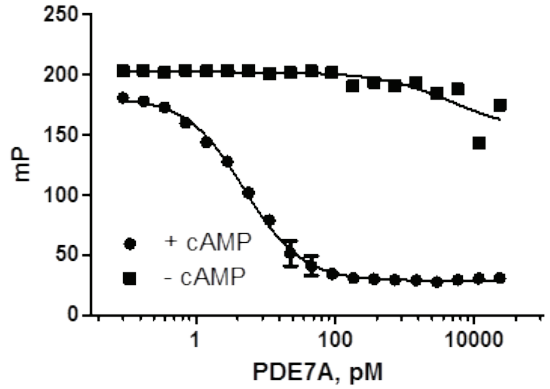


Figure 13. *Raw data of PDE7A enzyme titration. Control shown without cAMP substrate. EC_{80} of PDE7A was 5 pM.*

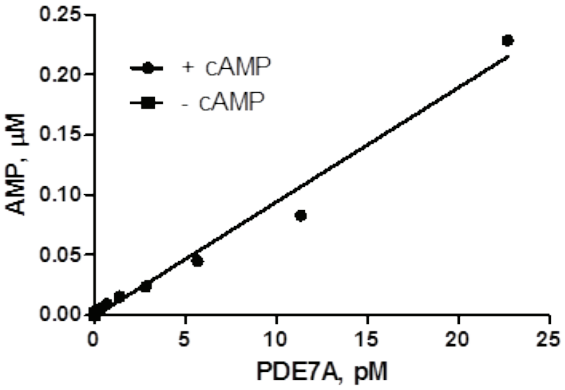


Figure 14. *Data converted to product formed shows linearity under initial velocity conditions when used at the EC_{80} of 5 pM.*

Next, dose-response curves were generated for each of the three inhibitors to determine IC_{50} values. A streamlined protocol for determination of residence time was applied, as per Examples 1 through 3:

- Preincubate 10 × [IC_{50}] compound + 100 × [EC_{80}] target
- Perform a 100X jump-dilution into a reaction mixture of cAMP and Transcreener detection reagents.
- Continuously read over time and monitor the change in fluorescence.
- Analyze data and calculate residence times.
- Preincubation conditions were as follows:

Enzyme for [EI]
100 × EC_{80} (PDE7A) = 5 pM × 100 = 500 pM

Inhibitor for [EI]

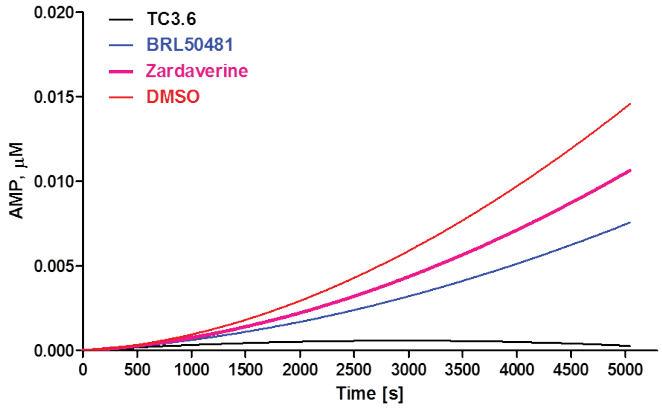
10 × IC_{50} (TC3.6) = 637 µM

10 × IC_{50} (BRL50481) = 169 µM

10 × IC_{50} (Zardaverine) = 100 µM

Preincubation mixes were prepared with PDE7A enzyme at a concentration of 100 × EC_{80}, to provide a robust signal after dilution, and three different inhibitors (TC3.6, BRL50481, Zardaverine) at 10 × IC_{50}, to ensure that the enzyme was saturated with the inhibitor. The mix was incubated for 1 hour at room temperature to ensure formation of the [EI] complex.

To perform the jump-dilution method, the dilution of the [EI] complex is such that the inhibitor concentration is at least 100-fold below its IC_{50} value. A 100-fold dilution (0.2 µL of PDE7A/inhibitor mixture into 19.8 µL of detection mixture) was made in an low volume 384 well plate. The detection mixture was comprised of 1 µM cAMP in buffer also containing 2 nM tracer and 1.4 µg/mL of AMP[2] antibody.



	TC3.6	BRL50481	Zardaverine
Koff	0.000009739	0.00003409	0.00005468
Tau(min)	1.62317E-07	5.68167E-07	9.11333E-07

Figure 15. *Residence times of three inhibitors based on product formed of the enzyme PDE7A using the integrated rate equation.*

The plate was mixed well and read kinetically every 5 minutes for 4 hours in a Tecan Safire plate reader using the fluorescent polarization mode with EXC at 630 nm and EMS at 670 nm. Residence time data are shown in the Figure 15, with TC3.6 showing the shortest residence time compared to the other inhibitors BRL50481 and Zardaverine.

High-Throughput Drug Residence Time Screening Using Jump-Dilution Kinetics

Traditional protocols for determining inhibitor reversibility and estimating off-rates using jump-dilution assays involve manipulating drug concentration by a factor of its IC_{50}, which is predicated on having prior endpoint data available. As an alternative, it is possible to use an enzyme concentration to cover a wide range of inhibitor affinities while keeping inhibitor concentration constant. Researchers at EMD Serono presented this data in a poster with scientists from BellBrook Labs.[15]

The Labcyte® Echo 555 instrument was used to enable rapid and low volume transfer of aqueous solutions to measure residence times in an HTS format. By preincubating the enzyme and inhibitor reaction in an Echo-qualified source plate, a small volume from each well can be transferred to an assay plate. The enzyme inhibitor complex is diluted 1000-fold and recovery of enzyme activity is observed by reading fluorescence intensity in kinetic mode. Using this method, over 350 inhibitors can be profiled in a single experiment on one 384 well plate.[15]

Methods

- The Transcreener ADP[2] FI Assay was used to study potential inhibitors of a serine/threonine kinase.
- Compounds from a library were plated at a single concentration in an Echo-qualified source plate.
- Buffer solution with 1000X screening concentration of enzyme was added to all wells with automated liquid handler and incubated for 90 minutes
- 20 nL of enzyme-inhibitor solution was replicated from the source plate to a 384-well assay plate.
- A buffer solution containing ATP, peptide substrate, DTT, cofactor, and Transcreener ADP[2] reagents was added using an automated liquid handler, diluting the 1000X enzyme concentration down to 1X.
- The plate was read immediately in kinetic mode.
- Raw data was converted to a time course measurement.

Potential slowly-dissociating inhibitors were identified using a formula that compares each compound's kinetics to control wells. Outputted well selections were mapped to internal compound name and verified manually.

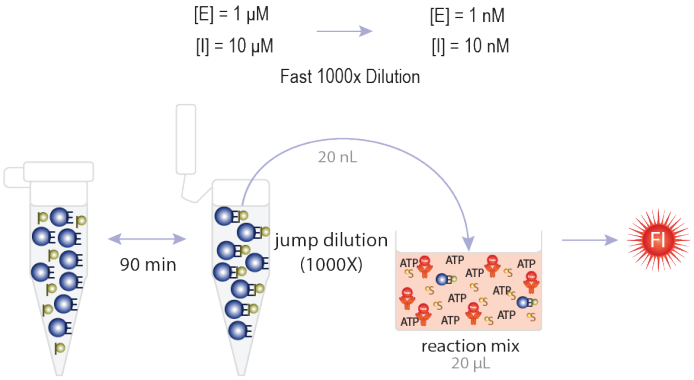


Figure 16. *Protocol for determining residence times using a jump-dilution assay in an HTS format.*

For each inhibitor showing slow recovery of enzymatic activity, the rate of return after dilution was fitted to the following equation:

$$[P] = v_s t + \frac{v_i - v_s}{k_{obs}} [1 - \exp(-k_{obs} t)]$$

Where V_i and V_s are the initial and steady-state rates of the reaction in the presence of inhibitor, k_{obs} is the apparent first order rate constant for the transition from V_i to V_s, and t is time.

Under the experimental conditions, k_{obs} approximates the dissociation rate constant (k_{off}) of the enzyme–inhibitor complex, and therefore allows inhibitor residence time to be estimated as $1/k_{obs}$. Using this technique, two potential inhibitors were identified (Compound A and Compound B).

	IC_{50} (nM)	K_{obs} (1/s)	Residence Time (min)
Compound A	13	0.0006	28
Compound B	13	>0.1	<1

Table 4. *Kinase inhibitor residence times determined in an HTS format using the raw data from Figure 17 and the equation from this example.*

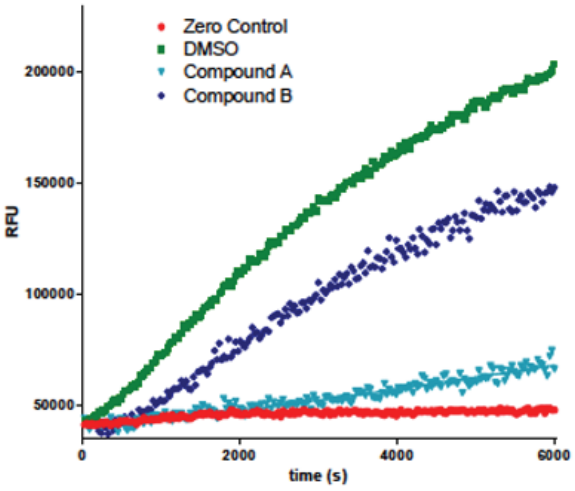


Figure 17. *Raw data illustrating residence times of two inhibitors with a kinase enzyme. Data provides a graphical representation of approximate residence time to rank order compounds for lead discovery in an HTS format.*

Using this data, residence time was estimated in such a way that each compound in the plate could be rank ordered. Further research can then be done on each individual compound to accurately measure the residence time using the formats from Examples 1-4.

Conclusion

Jump-dilution assays offer a HTS-compatible, generic method that facilitates the use of residence time as a parameter for compound prioritization and optimization early in drug discovery programs. Transcreener is a competitive immunoassay format that enables homogenous detection of nucleotides with FP, FI, or TR-FRET readouts. The assay format is well-suited for the jump-dilution method because the detection reagents can be added directly to enzyme reactions to monitor recovery of activity. The technique is broadly applicable to target families. For example, when used for kinases, detection of ADP via Transcreener assays is suitable for any kinase family member regardless of its acceptor substrate. The Transcreener-based jump dilution method provides a streamlined approach for prioritizing hits based on residence time and uses existing HTS infrastructure.

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Associated Transcreener Assay Kits

Assay Kit	Kit Size	Catalog Number
Transcreener ADP² FP	1,000 Assays	3010-1K
Transcreener ADP² FP	10,000 Assays	3010-10K
Transcreener ADP² FI	1,000 Assays	3013-1K
Transcreener ADP² FI	10,000 Assays	3013-10K
Transcreener ADP² TR-FRET	1,000 Assays	3011-1K
Transcreener ADP² TR-FRET	10,000 Assays	3011-10K
Transcreener AMP²/GMP² FP	1,000 Assays	3015-1K
Transcreener AMP²/GMP² FP	10,000 Assays	3015-10K
Transcreener AMP²/GMP² TR-FRET	1,000 Assays	3020-1K
Transcreener AMP²/GMP² TR-FRET	10,000 Assays	3020-10K
Transcreener UDP² FP	1,000 Assays	3018-1K
Transcreener UDP² FP	10,000 Assays	3018-10K
Transcreener UDP² TR-FRET	1,000 Assays	3022-1K
Transcreener UDP² TR-FRET	10,000 Assays	3022-10K

*Kit sizes are based on a 384 well format

Learn more about using biochemical assays to determine residence time by going to bellbrooklabs.com and searching keyword “residence time.” There you will find more application notes, recorded webinars, tutorials, and examples of residence time being measured with Transcreener assays.