Application Note

BellBrook Labs

# Determination of Drug-Kinase Residence Time with the Transcreener ADP² FP Assay

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## Introduction

This Application Note provides a protocol and experimental example for the use of the Transcreener® ADP² FP Assay to determine the residence time of a drug (or drug candidate) during its interaction with a kinase.

The duration of residence time may be highly relevant for assessing the durable pharmacologic effects of a drug candidate. In general, longer residence time results in improved efficiency, as the extended contact between drug and enzyme results in extended inhibition of enzyme activity. This in turn allows pharmacological effect to be achieved over protracted time at lower doses, reducing off-target effects. For this reason, the determination of residence time is highly informative.

Residence time ($\tau$) is the time that a drug remains bound to its target before dissociating. Residence time is the reciprocal of dissociation rate ($k_{off}$).

The dissociation rate constant $k_{off}$ can be determined using a "jump dilution" method in which enzyme activity is monitored over time as an inhibitor dissociates. The jump dilution method is performed in four steps:

- STEP 1. Incubate the kinase with a saturating concentration of inhibitor (e.g., 10 x $IC_{50}$).
- STEP 2. Dilute the kinase/inhibitor mixture into a solution containing all enzyme reaction components and Transcreener ADP² detection reagents.
- STEP 3. Measure fluorescent signal periodically to monitor the recovery of kinase activity over time.
- STEP 4. Determine $k_{off}$ by fitting enzyme progress curves to an integrated rate equation: [ $y = v_s \cdot t + (v_i - v_s/k_{obs})(1 - e^{-k_{obs} \cdot t/k_{obs}})$]

## Materials

- Black, Non-Binding, LV volume 384 well plate (Catalog # 4514, Corning)
- Transcreener ADP² 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)

## 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 application note; however, an example is given below.

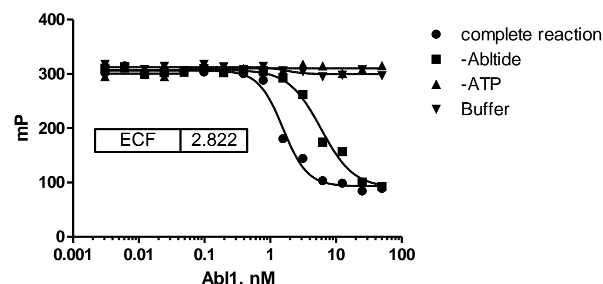
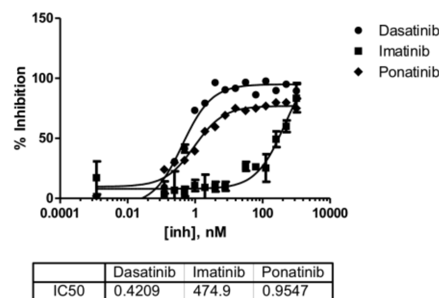



**Figure 1. Abl1 enzyme titration in the presence of 1 µM ATP and 10 µM Abltide. A concentration ($EC_{80}$) of 2.8 nM was determined to be optimal based on this titration. The reaction was carried out in buffer containing 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, and 0.01% Brij.**




| | Dasatinib | Imatinib | Ponatinib |
|---|---|---|---|
| IC50 | 0.4209 | 474.9 | 0.9547 |

**Figure 2. A dose response curve for Abl1 enzyme (2.8 nM) in the presence of 1 µM ATP and 10 µM Abltide. $IC_{50}$ values of 0.42 nM (Dasatinib), 475 nM (Imatinib), and 0.95 nM (Ponatinib) were determined based on this titration. The reaction was carried out in buffer containing 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, and 0.01% Brij.**

### II. Jump dilution method

#### II-a. Preincubation protocol

i) Select an amount of kinase for the pre-incubation step such that, after dilution in the binding assay, the resulting concentrations of kinase and antibody will still provide a robust signal. We recommend using a kinase concentration equivalent to the $EC_{80}$ value x 10.

ii) In general, the inhibitor concentration chosen should be 5- to 20-fold $IC_{50}$.

iii) For the example described above with Abl1, we choose 30 nM enzyme, 10 nM Dasatinib, 50 nM Ponatinib, and 5 µM Imatinib. DMSO alone controls (no inhibitor) were also run simultaneously. A no enzyme control is also critical to allow calculation of maximum inhibition.

iv) 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**

i) Ideally, the dilution step should bring the concentration of inhibitor below its $IC_{50}$ value. A typical dilution is 0.5 µL of Abl/Inhibitor mixture into 19.5 µL of Detection Mixture in a LV-384 well plate (Corning 4514, black). A 40-fold dilution was used in this example. Higher dilutions will yield better results.

   (1) Note: this method is called "jump dilution" because the [EI] complex is diluted in a detection mixture that contains saturating amounts of substrate.

ii) For Abl1, we used 5 µM ATP and 20 µM Abltide in kinase buffer containing 2 nM tracer and 5 µg/mL of ADP2 antibody.

iii) 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.

**II-c. Data analysis**

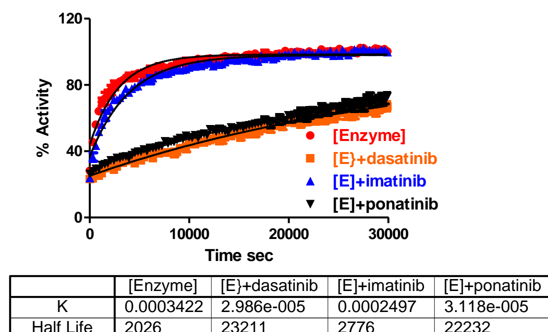
Data analysis was performed using the Graph Pad Prism software package.

i) Convert the polarization data into % Activity by setting the values obtained for the no inhibitor control as "100% active" and the values obtained for the no kinase control as "0% active."

ii) Based on this normalization, all mP values are expressed as % activity.

iii) Under the analysis tab, choose "one phase decay." The non-linear regression analysis window gives the $k_{off}$ rates and the half-lives.

**Residence time for Abl1 inhibitors using the Jump dilution method with Transcreener FP assay**




| | [Enzyme] | [E]+dasatinib | [E]+imatinib | [E]+ponatinib |
|---|---|---|---|---|
| K | 0.0003422 | 2.986e-005 | 0.0002497 | 3.118e-005 |
| Half Life | 2026 | 23211 | 2776 | 22232 |

**Figure 3. Residence times for Dasatinib, Ponatinib and Imatinib were determined for Abl1 in a jump dilution experiment using the Transcreener ADP[2] Assay. For Abl, compound incubations were performed with an Abl concentration of 30 nM and inhibitor concentrations of 5 nM (Dasatinib), 5 µM (Imatinib), or 10 nM (Ponatinib). A 40-fold jump dilution was performed by adding 19.5 µL of 20 µM Abltide and 5 µM ATP in presence of detection reagents.**

## Summary

We have described a jump dilution protocol for determining the dissociation rate constant $k_{off}$ using the Transcreener ADP[2] Assay. As an experimental example, residence times for Dasatinib, Ponatinib, and Imatinib for the target Abl1 are calculated.

## Ordering Information

Please visit www.bellbrooklabs.com or contact BellBrook Labs for pricing for the Transcreener® Assays.
Custom quotes are available for bulk orders.

Phone Orders: 608.443.2400

Toll-Free: 866.313.7881

Fax Orders: 608.441.2967

## Technical Information

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