Transcreener® ADP² Assays: Streamlining Inhibitor Profiling for Kinases with Diverse ATP Requirements.

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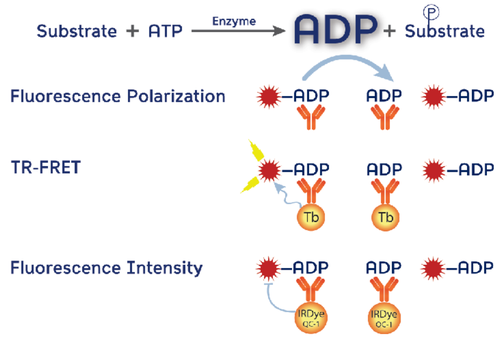
Abstract

The Transcreener ADP² Assays use direct immunodetection of ADP with three different fluorescent detection modes (FP, TR-FRET and FI) to provide the most sensitive, versatile HTS method for kinases and other ATP-utilizing enzymes. Transcreener is the only method that does not rely on the use of multiple coupling enzymes to convert the ADP product to a secondary analyte. Because it relies on a competitive binding reaction, Transcreener assays can be tuned to produce very good signal windows (Z' > 0.7) with ATP levels as low as 100nM and as high as 1mM, even at low percent conversion. This facilitates the profiling of inhibitors across diverse kinases using ATP at the corresponding K_m concentrations. Here we outline a streamlined approach for profiling kinase inhibitors, including optimization of enzyme concentration for initial velocity kinetics and maximal signal window, determination of IC_{50} values directly from raw fluorescence signal and examination of inhibitor mechanism. All of the methods outlined are consistent with HTS assay guidelines set forth by the NIH Chemical Genomics Center, and adhere to Michaelis-Menten kinetics assumptions. In addition, we show the equivalency of inhibitor potency determinations using the three different Transcreener detection formats. Together these methods will enable streamlined profiling of kinase inhibitors for enzymes with a range of ATP requirements.

Transcreener ADP² Assays: Direct immunodetection of ADP in three fluorescent detection modes



Steps for Streamlining Inhibitor Screening

1) Determine the K_m of ATP for target Enzyme from literature or by running experiment.

2) Calculate the optimal antibody concentration from the linear equation (EZ Protocol)

3) Run enzyme titration at K_m of ATP and saturating concentrations of acceptor substrate (Fig 4)

4) Run inhibitor screens and dose dependency curves at EC_{60}-EC_{80} concentration of enzyme. (Fig 5)

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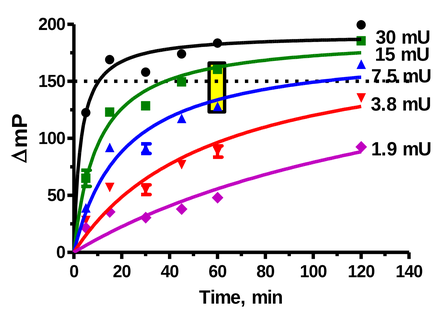

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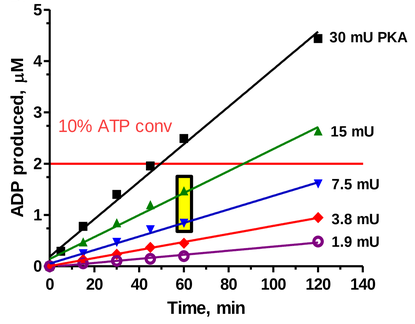

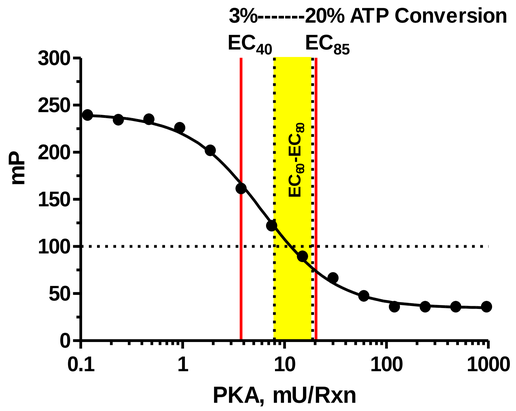

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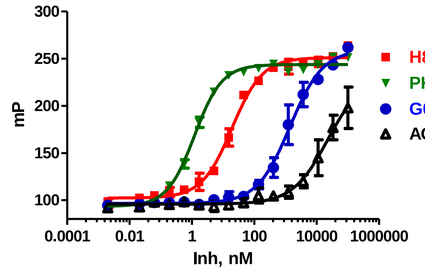

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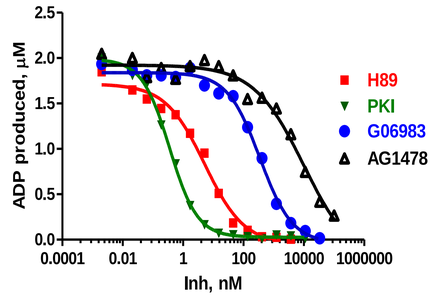


	H89	PKI	Go6983	Tyrophostin AGI 1478
IC_{50}- Raw Data (nM)	19.56	1.22	1405	>10000
IC_{50}- Converted Data (nM)	5.212	0.3583	349.7	>10000

Figure 3. Non linear response is normal for competitive binding assays A) Shows the mP shift , when PKA is titrated at various time points for generating the rates of reaction . B) Shows the same data converted to ADP formed (µM) . The enzyme reactions were run in the presence of 20µM ATP (K_m) and 20µM Kemptide. The highlighted portion represents EC_{60}-EC_{80} concentration of the target enzyme, the optimal concentration determined to run dose dependency curves for inhibitors. As shown in the converted data this range of enzyme concentration shows linearity and confirms that Transcreener assays follow the initial rates kinetics.

Figure 4. Determining optimal enzyme concentration for inhibitor profiling A) Shows the PKA titration curve at K_m of ATP, 20µM, and 20µM Kemptide after 1 hour incubation. The area within the two solid red lines represents 3%-20% ADP conversion and is the recommended concentration of enzyme to generate acceptable Z` (>0.7) for primary screens of inhibitors. The highlighted area within the curve shows the concentration of enzyme one would choose to perform a dose dependency inhibitor titration. This region is between EC_{60}-EC_{80} concentration (7-15mU PKA/Rxn) of the enzyme.

Figure 5. Determining IC_{50} values A) Shows the dose response curves of representative inhibitors of PKA generated using the EC_{70} concentration of PKA (12mU/Rxn) in the presence of 20 µM ATP (K_m) and 20 µM Kemptide . B) Shows the same dose response curves when the raw data is converted to ADP produced. The IC_{50} of the raw data is approximately 2.5 fold greater than the IC_{50} from the converted data.

Conclusions

- For Inhibitor dose dependency curves we recommend using EC_{60}-EC_{80} concentrations of target enzymes.
- There is a linear correlation between IC_{50} values determined from raw polarization data and ADP formation with a slope of 2.5
- We show that we can distinguish competitive and non competitive ATP inhibitors using Transcreener ADP² assay.
- Similar inhibitor potencies, K_m values and IC_{50} values were observed for the three detection modes of Transcreener assay.
- The process of inhibitor profiling can be streamlined with Transcreener ADP² assays by using the recommended concentrations of antibody.

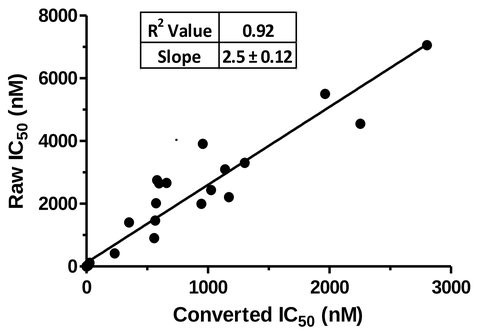
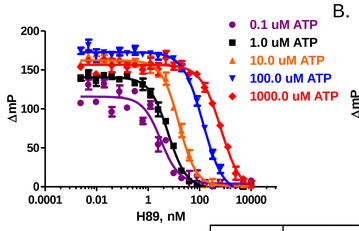


Figure 6. Linear correlation of IC_{50} values determined from raw polarization and ADP formation. The IC_{50} of raw data and IC_{50} of converted data was generated using EC_{60}, EC_{70} or EC_{80} concentrations of the target enzymes and reference inhibitors. Enzymes used in the study were PKA, AKT2, Rock1, Clk4, Zap 70 and Abl1 with ATP K_m being 20µM , 300µM, 2µM,4µM, 3µM and 4µM respectively. The inhibitors used were Staurosporine, K252a, H89, Tyrophostin AGI 1478, PKI and Gleevac. The slope of the line shows that the IC_{50} of the raw data is approximately 2.5- fold greater than the IC_{50} of the converted data.

A.



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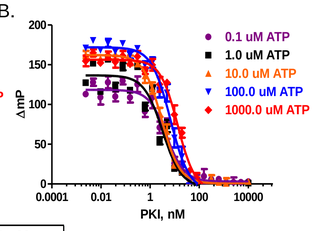
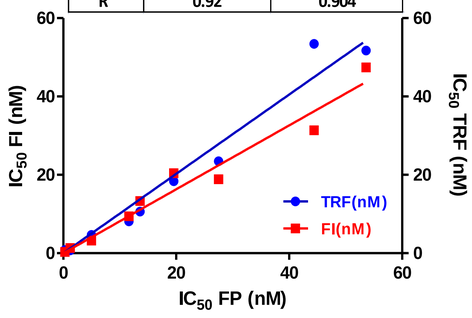


	IC50 (nM)	
ATP (µM)	H89	PKI
0.1	3	5
1	6	3
10	16	3
100	135	7
1000	675	13

Figure 7. Differentiating between competitive and non competitive inhibitors. A) Shows the dose response curves of ATP competitive inhibitor, H89 at different ATP concentrations using the Transcreener ADP² FP assay. The IC_{50} values across the different ATP concentrations changes from 3nM to 670nM confirming the competitive nature of the inhibitor. B) Shows the dose response curve of non ATP competitive inhibitor, PKI at different ATP concentrations using the Transcreener ADP² FP assay. The IC_{50} value across the various ATP concentrations remains relatively same, ranging from 4nM to 13nM .

	TRF(nM)	FI(nM)
Slope	1.013 ± 0.04938	0.8155 ± 0.03846
R^2	0.92	0.904



	FP	FI	TRF
Km of Kemptide for PKA (µM)	2	1.8	2.03
Km of ATP for PKA(µM)	23.65	16.7	20
EC50 of PKA (mU/Rxn)	6	10	5

Table 1. Equivalency of the three detection modes. Shows the three fluorescent detection modes generating similar K_m values, EC_{50} and IC_{50} values by using PKA and its inhibitors as an example.

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