Proposal of Substrate Competitive Study
- HotSpot Kinase Assay -

This proposal is to test whether the customer's compound is competitive with respect to substrate or not. The results include kinetic constants, K_m for substrate and K_i for the inhibitor, as well as mode of inhibition at a constant ATP concentration. The assay designed here is based on the previous data RBC performed for the customer. If the compound has not been tested with the target kinase by RBC, the IC_{50} determination is required prior to proposal.

Materials and reagents:

Compound information from the customer

Kinase reaction buffer: 20 mM HEPES-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.02% Brij35, 0.1 mM Na_3VO_4, 0.02 mg/ml BSA, 2 mM DTT, and 1% DMSO.

Kinases: (Example)
ROCK2; Recombinant Human protein (amino acids 5-554), N-terminal GST-tagged, expressed in Sf9 cells. Mw=88 kDa.

Substrate for kinase: (Example)
For ROCK2; Long S6 kinase substrate peptide,
[KEAKEKRQEQIAKRRRLSSLRASTSKSGGSQK], Mw=3,630 Da

Standard reaction conditions (unless otherwise specified):
1 nM ROCK2, 10 μM ATP (or customer's choice), and varied substrate

Experimental Procedures:

The kinase assay will be performed at room temperature. Compounds will be added 10-dose IC_{50} mode into Enzyme/substrate mixture using acoustic technology at 5 substrate concentrations, and pre-incubated for 20 min* to ensure compounds are equilibrated and bound to the enzyme. Then a constant concentration of ATP will be added to initiate the reaction. The activity will be monitored every 5-15 min for a time course study. Substrate and compound concentrations will be tested as follows:

1. K_i determination for kinase

Substrate concentrations will be tested: 1, 2, 5, 10, and 20 μM substrate at 10 μM ATP

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Compound concentrations will be tested: 10-dose IC_{50} with 3-fold serial dilution started at 10 μM.

Time points will be measured: 0, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, and 120 min

* Standard pre-incubation time is 20 min.

Data Analysis:

The reactions will be monitored every 5-15 min to obtain progress curves with time course. At each time point, radioisotope signal (^{33}P) will be converted into "μM phosphate transferred to substrate", and will be plotted against time. The slopes of initial linear portion of progress curves will be obtained by linear regression in Excel. The slopes (or velocity; μM/min) will be then plotted against substrate concentrations for Michaelis-Menten plot, and subsequent Lineweaver-Burk plot (double-reciprocal plot), using GraphPad Prism software. The results will be further analyzed by global fit using GraFit software with the "Mixed Inhibition" equation (1).

$$v = \frac{V_{max}.[S]}{K_m\left(1+\frac{[I]}{K_i}\right)+\left(1+\frac{[I]}{K_i'}\right)[S]} \quad (1)$$

Where v is velocity, [S] is substrate concentration, [I] is inhibitor concentration, K_i is inhibitor affinity for enzyme, and K_i' is inhibitor affinity for enzyme/substrate complex. High number (infinity) of K_i' values mean almost no inhibitor affinity for enzyme/ substrate complex, which turns pure competitive inhibition. If the compound is noncompetitive with respect to substrate, K_i and K_i' values will be equal.

The report in PDF file will contain detailed assay performed, data and figures, analyses results, and summary table of kinetic constants and mode of inhibition.

Limitations of this proposal:

1. This proposal is designed for "simple and reversible" inhibitors. If the inhibitor is as follows, further studies may be needed:
   a. Time dependent inhibitor: The progress curves will be non-linear, which makes the analyses difficult. Since the compound is pre-incubated for 20 min already, the curve fitting for on-rate will not be accurate. It needs different assay design for determination of on-rate (i.e., k observe).
   b. Tight-binding inhibitor: The tight-binding inhibitor binds to the enzyme at 1:1 molar ratio, and apparent IC_{50} or K_i will be lower than the enzyme concentration. It needs different assay design for K_i determination.

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