Proposal of Reversibility Study
- HotSpot Kinase Activity Assay -

This proposal is to test whether the customer's compound inhibits enzyme reversibly or not. The assay is designed for a non-tight binding inhibitor. The protocol in brief is; the enzyme at 100X concentration is incubated with compound (1:1 molar ratio), and then diluted 100-fold into the reaction buffer to initiate the reaction. The assay designed here is based on the previous data RBC performed for the customer. In this format, the IC_{50} value of the compound must be higher than the enzyme concentration in the reaction. **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): (Example)
1 nM ROCK2, 20 μM peptide substrate, and 10 μM ATP

Experimental Procedure:

Reversibility (Example)

The enzyme at 100X concentration is incubated with compound (1:1 molar ratio), and then diluted 100-fold into the reaction buffer to initiate the reaction.

Pre-incubation conditions: standard incubation for 20 min*

A. 100 nM ROCK2 + 100 nM Compound
B. 100 nM ROCK2 (No inhibitor control)

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Reaction conditions:

1. 1 nM ROCK2 + 1 nM Compound (Expecting >90% activity if reversible based on previous IC_{50} value of 100 nM)
2. 1 nM ROCK2 (from incubation B)
3. 1 nM ROCK2 (from incubation B) + 1 nM Compound (add fresh into the reaction without incubation: 0.01X IC_{50})

ATP concentration: 10 μM ATP

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

* Pre-incubation time can be longer if slow binding is expected.

Data Analysis:

If the compound is reversible, the activities of reaction conditions #1 and #3 will be the same level. If the compound is irreversible, the activity of reaction condition #1 will stay an inhibited level. If off rate is slow but less than 2 hours, the progress curve for the reaction #1 will be upward curve (non linear). Off rate will be estimated if the data points are enough for curve fit (up to 2 hours).

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 curve of reaction #3 will be non-linear, which makes the analyses difficult. It needs on-rate study first to find out pre-incubation time to ensure compound binding to enzyme for reversibility measurement.
   b. Tight-binding inhibitor: The tight-binding inhibitor binds to the enzyme at K_i lower than the enzyme concentration in the reaction. Therefore 100-fold dilution at 1:1 molar ratio may not work. It needs several concentrations of compound in pre-incubation to ensure residual enzyme activities can be measured.
2. The time frame of this proposal is 2 hours. If the off-rate is longer than 2 hours, reversibility cannot be observed.

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