Technical Bulletin

c-Src Kinase Inhibitor Screening Kit

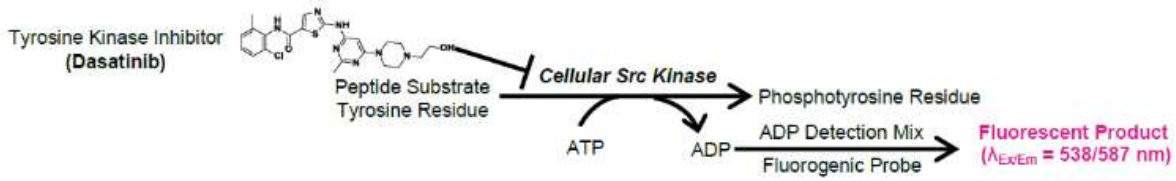
Catalog Number MAK426

Product Description

Cellular Src Kinase (c-Src) is a non-receptor tyrosine kinase that regulates a wide array of cellular signal transduction pathways by phosphorylation of specific tyrosine residues in other tyrosine kinases. Src kinase family members interact with many proteins such as receptor tyrosine kinases, GPCRs, ion channels, steroid receptors, transcription activators, solute transporters and transmembrane adhesion receptors. c-Src was the first "proto-oncogene" to be identified. Overexpression of wild type c-Src or expression of a constitutively active mutant form is frequently found in a number of different cancers. Activation of c-Src enhances angiogenesis, proliferation and invasion pathways in tumors. The extent of this activation typically correlates with the malignant potential and patient survival. Despite the significant role of c-Src in oncogenesis, there are no known selective c-Src inhibitors. A number of clinically used tyrosine kinase inhibitors are capable of inhibiting c-Src. However, the currently available drugs are not selective and inhibit multiple kinases. Selective c-Src inhibitors may be more efficacious and can exhibit fewer side effects than the promiscuous multi-kinase inhibitors.

The c-Src Kinase Inhibitor Screening Kit enables rapid screening of test compounds for modulation of c-Src activity. The assay uses a c-Src-specific polypeptide substrate and a high concentration of an ultra-pure ATP that closely reflects physiological ATP levels. ADP formation during the kinase reaction is measured. The strong and stable fluorescence signal (λ_{Ex} = 535 nm/ λ_{Em} = 587 nm) generated during the reaction is directly proportional to the amount of ADP generated. This ensures a high signal-to-background ratio and little interference due to short wavelength fluorescence by test compounds. The kinase inhibitor Dasatinib is included in the kit for use as a reference inhibitor to show the assay is performing appropriately. The assay is simple, highly sensitive, and suitable for high-throughput applications.

The kit is suitable for screening and characterization of drugs and novel chemical entities for inhibition or modulation of c-Src activity and for the development of structure-activity relationship models to predict Src kinase selectivity of compounds.



Components

The kit is sufficient for 100 fluorometric assays in 96-well plates.

- Src Assay Buffer — 25 mL
  Catalog Number MAK426A
- Fluorogenic Probe — 200 µL
  Catalog Number MAK426B
- ADP Detection Mix — 1 vial
  Catalog Number MAK426C
- Developer Enzyme Mix — 1 vial
  Catalog Number MAK426D
- Src Peptide Substrate — 1 vial
  Catalog Number MAK426E
- Ultra-Pure ATP — 1 vial
  Catalog Number MAK426F
- Recombinant Human c-Src — 100 µL
  Catalog Number MAK426G
- Dasatinib (1 mM) — 50 µL
  Catalog Number MAK426H

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (e.g., multichannel pipettor)
- Fluorescence multiwell plate reader
- White flat-bottom 96-well plates. Cell culture or tissue culture treated plates are **not** recommended.
- Dounce tissue grinder set (Catalog Number D9063 or equivalent)
- Microcentrifuge capable of RCF ≥13,000 × *g*

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on dry ice. Store components at -80 °C, protected from light.

Preparation Instructions

Briefly centrifuge small vials prior to opening

Src Assay Buffer: Allow to thaw to room temperature prior to use. Store at 2-8 °C, protected from light.

Fluorogenic Probe (in DMSO): Divide into aliquots and store at -20 °C, protected from light. Prior to use, warm the solution to room temperature. After use, promptly retighten cap to minimize adsorption of airborne moisture.

ADP Detection Mix and Developer Enzyme Mix: Reconstitute each vial with 220 µL of Src Assay Buffer. Aliquot as desired and store aliquots at -20 °C, protected from light. Avoid repeated freeze/thaw cycles.

Src Peptide Substrate: Reconstitute vial with 220 µL of purified water to obtain a 50× stock solution. Aliquot and store at -20 °C, protected from light. Stable for three freeze/thaw cycles.

Ultra-Pure ATP: Reconstitute vial with 220 µL of purified water to obtain a 12.5 mM stock solution. Aliquot and store at -80 °C. Avoid repeated freeze/thaw cycles. Keep thawed aliquots on ice while in use.

Recombinant Human c-Src: Aliquot as desired and store aliquots at -80 °C. Avoid repeated freeze/thaw cycles. Keep thawed aliquots on ice while in use.

Dasatinib (1 mM): Provided as a solution in DMSO. Allow to thaw to room temperature prior to use. Stable for three freeze/thaw cycles.

Procedure

All samples and standards should be run in duplicate.

Test Compound Preparation

1. For each Test Compound, dissolve in appropriate solvent to produce a stock solution.
2. Prepare a 10× working solution by diluting the stock solution from Step 1 in Src Assay Buffer.
3. To determine IC_{50} values for Test Compounds, 10× solutions should be diluted to a range of concentrations in order to generate a multi-point dose-response curve. NOTE: The concentration of organic solvent should be the same for all test concentrations.
4. To designated well(s), add 10 µL of each prepared Test Compound solution.
5. Add 40 µL of Src Assay Buffer to each well.

Solvent Control

Organic solvent concentration should be minimized to avoid impacting enzyme activity (DMSO has little effect on c-Src kinase activity at a final concentration of ≤1%). For higher concentrations or solvents other than DMSO, prepare a Solvent Control well with the same final concentration of the organic solvent used to solubilize test compounds. Use the Solvent Control well to define 100% activity if the RFU readings are significantly different from the No Inhibitor Control well(s).

To designated well, add 50 µL of Src Assay Buffer containing the test compound solvent at a 2× final well concentration.

Reference Inhibitor Control

1. Dilute the 1 mM Dasatinib stock solution at a 1:100 ratio by adding 10 µL of the 1 mM solution to 990 µL of Src Assay Buffer, yielding a 10 µM Dasatinib working solution.
2. To designated well(s), add 10 µL of the working solution to each Reference Inhibitor Control well.
3. Add 40 µL of Src Assay Buffer to each well.

No Inhibitor Control

Add 50 µL of Src Assay Buffer to designated well(s).

Background Control

Add **60 µL** of Src Assay Buffer to designated well(s).

Assay Reaction Preparation

1. Mix enough reagents for the number of assays to be performed. For each Test Compound, Solvent Control, Reference Inhibitor Control, and No Inhibitor Control well, prepare 10 µL of Diluted c-Src Enzyme Preparation according to Table 1. Mix well.

Table 1.
Diluted c-Src Enzyme Preparation

Reagent	Volume
Recombinant Human c-Src	1 µL
Src Assay Buffer	9 µL

2. Add 10 µL of Diluted c-Src Enzyme Preparation to each Test Compound, Solvent Control, Reference Inhibitor Control, and No Inhibitor Control well. **Do not add** diluted c-Src Enzyme to Background Control well(s).
3. Pre-incubate the plate for 15 minutes at 37 °C to allow Test Compound(s) to interact with c-Src. During the pre-incubation, prepare Reaction Mix.

Reaction Mix

1. Mix enough reagents for the number of assays to be performed. For each well, prepare 40 µL of Reaction Mix according to Table 2. Mix well.

Table 2.
Reaction Mix Preparation

Reagent	Volume
ADP Detection Mix	2 µL
Developer Enzyme Mix	2 µL
Ultra-Pure ATP	2 µL
Src Peptide Substrate	2 µL
Fluorogenic Probe	1 µL
Src Assay Buffer	31 µL

2. Start the reaction by adding 40 µL of Reaction Mix to all reaction wells, yielding a final reaction volume of 100 µL/well.

Measurement

Measure the fluorescence (RFU) at λ_{Ex} = 535 nm/ λ_{Em} = 587 nm in kinetic mode at 37 °C for 30-45 minutes. While the assay can be performed in either endpoint or kinetic mode, it is strongly recommended to read in kinetic mode in order to ensure that the measurements recorded are within the linear range of the reaction.

Results

1. For each reaction well (including Background Control, No Inhibitor Control, and Solvent Control wells), choose any two time points (T_1 and T_2) in the linear phase of the reaction progress curve.
2. Obtain the corresponding fluorescence values at those points (RFU_1 and RFU_2) and determine $\Delta F = (RFU_2 - RFU_1)$ and $\Delta T = (T_2 - T_1)$.
3. Calculate the Background-corrected reaction rate (denoted by R) for each well by subtracting the rate of the Background Control (ΔF_{BC}) reaction from each reaction rate:

   $R_{TC} = (\Delta F_{\text{Test Compound}} - \Delta F_{BC}) / \Delta T$

   $R_{SC} = (\Delta F_{\text{Solvent Control}} - \Delta F_{BC}) / \Delta T$

   $R_{NI} = (\Delta F_{\text{No Inhibitor}} - \Delta F_{BC}) / \Delta T$

   $R_{RI} = (\Delta F_{\text{Reference Inhibitor}} - \Delta F_{BC}) / \Delta T$

4. Calculate the percent inhibition due to the Test Compound (or Reference Inhibitor Control) using the following equation where R_{RI} represents the Background-corrected rate of the No Inhibitor Control:

$$\% \text{ Relative Inhibition} = \frac{R_{NI} - R_{TC}}{R_{NI}} \times 100\%$$

5. If the Solvent Control RFU readings are significantly different from the No Inhibitor Control RFU readings, calculate the percent inhibition due to the Test Compound (or Reference Inhibitor Control) using the following equation where R_{SC} represents the Background-corrected rate of the Solvent Control:

$$\% \text{ Relative Inhibition} = \frac{R_{SC} - R_{TC}}{R_{SC}} \times 100\%$$

Figure 1.
Reaction kinetics of recombinant c-Src enzyme at 37 °C in the presence and absence of the indicated tyrosine kinase inhibitors, including the potent Abl/Src-family kinase inhibitor (Dasatinib) and a more promiscuous tyrosine kinase inhibitor (PP2). The Vehicle Control (Solvent Control) reaction contained assay buffer with a final concentration of 0.1% DMSO.

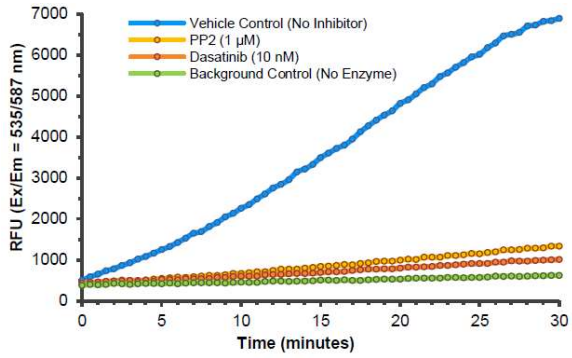
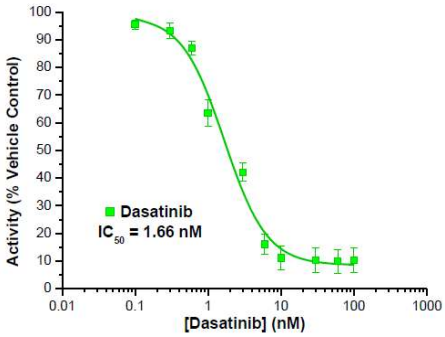


Figure 2.
Dose-response curve for the Reference inhibitor (Dasatinib). Percent activity was calculated for each concentration of the inhibitor by comparing with the activity of reactions containing No Inhibitor. IC_{50} value was derived by 4-parameter logistic curve fitting with each point representing the mean ± SEM of at least four replicates. Assays were performed according to the kit protocol.



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