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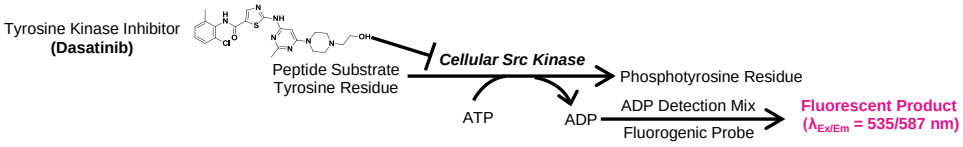
c-Src Kinase Inhibitor Screening Kit

rev 03/21

(Catalog # K2015-100; 100 Reactions; Store at -80°C)

I. Introduction:

Cellular Src Kinase (c-Src, EC 2.7.10.2) 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. Over-expression 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. **BioVision's 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 the ultra-pure ATP that closely reflects the physiological ATP levels. ADP formation during the kinase reaction is measured. The strong and stable fluorescence signal (Ex/Em = 535/587 nm) generated during the reaction is directly proportional to the amount of ADP generated. This ensures a high signal-to-background ratio and a little interference due to short wavelength fluorescence by test compounds. The assay is simple, highly sensitive and is high-throughput adaptable. The kit contains a complete set of reagents sufficient for performing 100 reactions in a 96-well plate format.



II. Applications:

- Screening and characterization of drugs and novel chemical entities for inhibition or modulation of c-Src activity.
- Development of structure-activity relationship models to predict Src kinase selectivity of compounds.

III. Kit Contents:

Components	K2015-100	Cap Code	Part Number
Src Assay Buffer	25 ml	WM	K2015-100-1
Fluorogenic Probe	200 µl	Blue	K2015-100-2
ADP Detection Mix	1 vial	Purple	K2015-100-3
Developer Enzyme Mix	1 vial	Red	K2015-100-4
Src Peptide Substrate	1 vial	White	K2015-100-5
Ultra-Pure ATP	1 vial	Orange	K2015-100-6
Recombinant Human c-Src	100 µl	Green	K2015-100-7
Dasatinib (1 mM)	50 µl	Amber	K2015-100-8

IV. User Supplied Reagents and Equipment:

- Multiwell fluorescence microplate reader
- Black 96-well plate with flat bottom

V. Storage Conditions and Reagent Preparation:

Prior to use, store kit at -80°C and protect from light. Briefly centrifuge all small vials prior to opening. Read the entire protocol before performing the assay procedure.

- **Src Assay Buffer:** Allow to thaw to room temperature (RT) before use. Store at 4°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 RT. After use, promptly retighten cap to minimize adsorption of airborne moisture.
- **ADP Detection Mix** and **Developer Enzyme Mix:** Reconstitute each vial with 220 µl Src Assay Buffer. Aliquot as desired and store aliquots at -20°C, protected from light. Avoid repeated freeze/thaw cycles.
- **Src Peptide Substrate:** Reconstitute with 220 µl ddH_2O to obtain a 50X stock solution. Aliquot and store at -20°C, protected from light. Stable for 3 freeze/thaw cycles.
- **Ultra-Pure ATP:** Reconstitute with 220 µl ddH_2O 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 RT before use. Stable for 3 freeze/thaw cycles.

VI. c-Src Inhibitor Screening Assay Protocol:

1. **Test Compound Preparation:** For each Test Compound (TC), dissolve in proper solvent to produce a stock solution and prepare a 10X working solution by diluting the stock solution in Src Assay Buffer. To determine IC_{50} values for TCs, 10X solutions should be prepared in a range of concentrations in order to generate a multi-point dose-response curve (*the concentration of organic solvent should be the same for all test concentrations*). 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,

we recommend preparing a Vehicle Control (VC) well with the same final concentration of the solvent used to solubilize TCs. Use the VC well to define 100% activity if it is different from the "No Inhibitor Control well(s)".

2. **Assay Reaction Preparation:**

   a. Prepare a diluted c-Src enzyme solution by diluting Recombinant Human c-Src stock in Src Assay Buffer at a 1:10 ratio (for example, for 10 reactions, mix 10 µl of Recombinant Human c-Src with 90 µl Src Assay Buffer). Make a sufficient amount of diluted c-Src enzyme to add 10 µl to each reaction well. **Note:** Remember to account for No Inhibitor Control/Vehicle Control and Reference Inhibitor Control wells when calculating the amount of diluted enzyme to prepare.

   b. Set up the Assay Reactions according to the table below. Prepare reaction wells containing **Test Compound(s)**, as well as corresponding **No Inhibitor Control** (which may also serve as the Vehicle Control (VC), if desired) and **Background Control** (containing no kinase). A **Reference Inhibitor Control** well may also be prepared using the included inhibitor Dasatinib. Dilute the 1 mM Dasatinib stock solution at a 1:100 ratio (add 10 µl of the 1 mM solution to 990 µl Src Assay Buffer), yielding a 10 µM Dasatinib working solution and add 10 µl of the working solution to each Reference Inhibitor Control well. Adjust the volume of all wells to 60 µl with Src Assay Buffer.

	No Inhibitor/VC	+Test Compound	Reference Inhibitor	Background Control
Diluted c-Src Enzyme	10 µl	10 µl	10 µl	—
Test Compound (10X)	—	10 µl	—	—
Dasatinib (10 µM)	—	—	10 µl	—
Src Assay Buffer	50 µl	40 µl	40 µl	60 µl

Note: For Vehicle Control (VC) well, use 50 µl Src Assay Buffer containing the test compound **solvent** at 2X final well concentration.

   c. Pre-incubate the plate for 15 min at 37°C to allow Test Compound(s) to interact with c-Src. During the pre-incubation, prepare Reaction Mix according to the table below. Make a sufficient amount of Reaction Mix to add 40 µl to each reaction well.

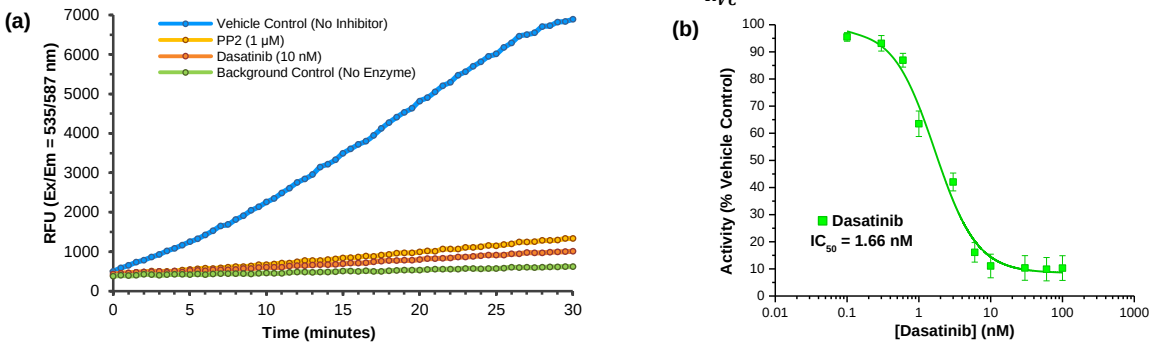
	Reaction Mix
ADP Detection Mix	2 µl
Developer Enzyme Mix	2 µl
Ultra-Pure ATP	2 µl
Src Peptide Substrate	2 µl
Fluorogenic Probe	1 µl
Src Assay Buffer	31 µl

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

3. **Measurement:** Measure the fluorescence at Ex/Em = 535/587 nm in kinetic mode at 37°C for 30-45 min. While the assay can be performed in either endpoint or kinetic mode, we strongly recommend reading in kinetic mode in order to ensure that the measurements recorded are within the linear range of the reaction.

4. **Calculation:** For each reaction well (including Background and No Inhibitor/Vehicle Control wells), choose any two time points (T_1 and T_2) in the linear phase of the reaction progress curve. Obtain the corresponding fluorescence values at those points (RFU_1 and RFU_2) and determine $\Delta F = (RFU_2 - RFU_1)$ and $\Delta T = (T_2 - T_1)$. 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: $\boldsymbol{R = (\Delta F - \Delta F_{BC}) / \Delta T}$. Calculate the percent inhibition due to the Test Compound or Reference Inhibitor Control using the following equation (where R_{VC} represents the Background-corrected rate of the No Inhibitor/Vehicle Control condition):

$$\textbf{\% Relative Inhibition} = \frac{R_{VC} - R_{TC}}{R_{VC}} \times 100\%$$



Figures: (a) 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 reaction contained assay buffer with a final concentration of 0.1% DMSO. **(b)** 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.

VII. Related Products:

ADPSensor Universal Kinase Activity Assay Kit (K212)
DiscoveryPak™ Receptor Tyrosine Kinase Inhibitor Set (K859)
Saracatinib (1582)
EZSolution™ Dasatinib (2029)

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