Omnia® Kinase Assay Kit User Guide

Catalog no: Various (see page 3) **Shipping: Dry Ice** **Storage: –20°C**

Protocol part no. PR322pps Rev. date: 31 March 2009

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1. Introduction

Protein kinases make up one of the largest human gene families, with 518 human protein kinases identified. As critical components of cellular signal transduction cascades, these enzymes regulate many essential biological mechanisms, including proliferation, differentiation, metabolism, and cell growth. A wide variety of human diseases have been linked to aberrant kinase activity. The ability to determine kinase activity and the effects of compounds that modulate this activity is essential for the development and screening of potential drugs against these enzymes.

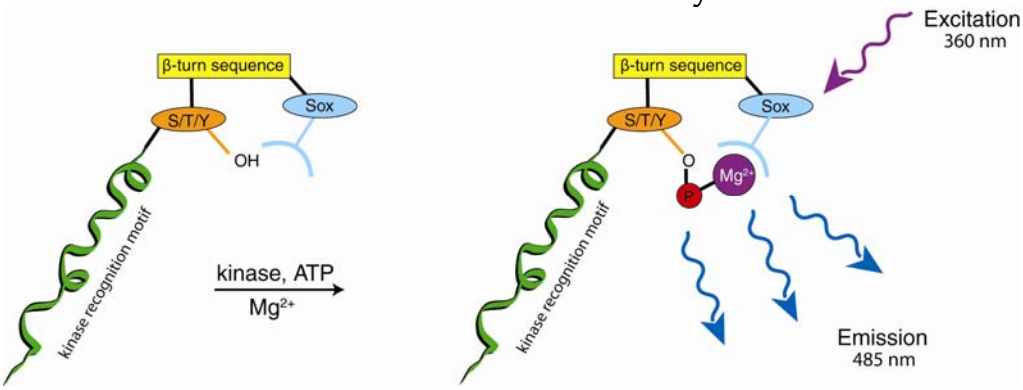
Omnia® Kinase Assays provide a fluorescence peptide substrate-based assay for the rapid, homogeneous, and sensitive detection of serine/threonine or tyrosine kinase enzymatic activity. Omnia® kinase assays can be used for a variety of applications, including kinetic studies, IC_{50} determinations, and kinase inhibitor screening. Forty-six different peptide substrates are available that can be used to measure the kinase activity of 193 different kinases (see reactivity table at www.invitrogen.com/omnia). Using these assays, you can measure kinase reactions under optimal kinetics, physiological (mM) ATP, and in real time without the use of radioactive tracers or specialized equipment. Omnia® assays directly measure the activity of the target enzyme without use of beads or secondary detection steps involving antibodies or enzymes. Because the assay can be performed with a wide range of ATP concentrations, including physiological (mM) levels, Omnia® assays can be used to select for both ATP competitive and ATP non-competitive (allosteric) kinase inhibitors.

2. Assay Principle

Omnia® Kinase Assays use the chelation-enhanced fluorophore (CHEF) 8-hydroxy-5-(N,N-dimethylsulfonamido)-2-methlyquinoline (referred to as Sox[1,2]). Sox is an unnatural amino acid that can be prepared as an Fmoc-protected derivative. This is incorporated into the substrate peptide using standard solid-phase peptide chemistry.

Upon phosphorylation of the peptide by a kinase, Mg^{2+} is chelated to form a bridge between the Sox moiety and the incorporated phosphate group on the serine, threonine, or tyrosine residue within the peptide (Figure 1). This results in an increase in fluorescence when the kinase reaction mixture is excited at 360 nm and the emission is measured at 485 nm, as shown in the following figure.

A. Mechanism of the Assay



B. Emission Spectra

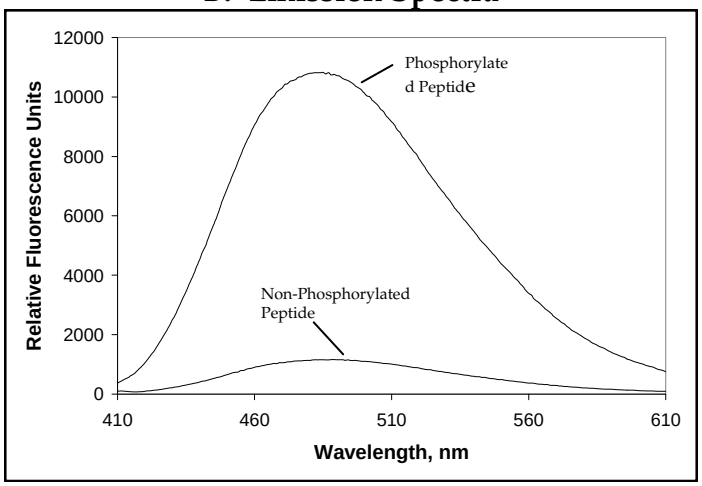


Figure 1: A. Schematic view of Mg^{2+} chelation by Sox and the phosphate group on a phosphorylated serine, threonine, or tyrosine residue. **B.** Fluorescence emission spectra of a Sox peptide substrate (lower) and a Sox phosphopeptide product (upper) in the presence of $MgCl_2$ generated using a constant excitation wavelength of 360 nm. The non-phosphorylated version of the Sox-modified peptide has a very low affinity for Mg^{2+} (K_d = 100–300 mM). The affinity for Mg^{2+} increases dramatically upon phosphorylation (K_d = 4–20 mM). Therefore, upon phosphorylation, most of the phosphopeptide exists in the Mg^{2+}-chelated, fluorescent state in the presence of $MgCl_2$.

3. Kits and Components

This user guide covers the following kits:

Omnia® Kinase Assay Kit	Catalog no.
Ser/Thr 1	KNZ1011
Ser/Thr 2	KNZ1021
Ser/Thr 3	KNZ1031
Ser/Thr 4	KNZ1041
Ser/Thr 5	KNZ1051
Ser/Thr 6	KNZ1061
Ser/Thr 7	KNZ1071
Ser/Thr 8	KNZ1081
Ser/Thr 9	KNZ1091
Ser/Thr 10	KNZ1101
Ser/Thr 11	KNZ1111
Ser/Thr 12	KNZ1121
Ser/Thr 13	KNZ1131
Ser/Thr 14	KNZ1141
Ser/Thr 15	KNZ1151
Ser/Thr 16	KNZ1161
Ser/Thr 17	KNZ1171
Ser/Thr 18	KNZ1181
Ser/Thr 19	KNZ1191
Ser/Thr 20	KNZ1201
Ser/Thr 21	KNZ1211
Ser/Thr 22	KNZ1221
Ser/Thr 23	KNZ1231

Omnia® Kinase Assay Kit	Catalog no.
Ser/Thr 24	KNZ1241
Ser/Thr 25	KPZ1251
Ser/Thr 26	KPZ1261
Ser/Thr 27	KNZ1271
Ser/Thr 28	KNZ1281
Ser/Thr 29	KPZ1291
Ser/Thr 30	KPZ1301
Ser/Thr 31	KPZ1311
Ser/Thr 32	KNZ1321
Tyr 1	KNZ3011
Tyr 2	KNZ3021
Tyr 3	KNZ3031
Tyr 4	KNZ3041
Tyr 5	KNZ3051
Tyr 6	KNZ3061
Tyr 7	KNZ3071
Tyr 8	KNZ3081
Tyr 9	KNZ3091
Tyr 10	KNZ3101
Tyr 11	KNZ3111
Tyr 12	KPZ3121
Tyr 13	KNZ3131
Tyr 14	KPZ3141

Each kit provides sufficient reagents to run two 384-well assay plates at a 20-µL final assay volume.

Catalog Number	Component	Description	Amount
Various	Omnia® Peptide	Omnia® peptide, 1 mM	220 µL
AS001A	ATP Solution	100 mM ATP in 20 mM Tris, pH 7.5	1 mL
P2325	DTT Solution	1 M DTT in water	1 mL
KB001A or KB002A	Kinase Reaction Buffer (10X)	Proprietary buffer, pH 7.5	10 mL

4. Materials Required but Not Supplied

- Recombinant kinase of interest (visit www.invitrogen.com/kinases for a list of Invitrogen kinases).
- Fluorescence microplate reader capable of reading λ_{ex} 360/λ_{em} 485 in a kinetic manner.

Note: Omnia® assays have been run on instruments such as the Tecan Safire 2™, Infinite® M1000, Infinite® F500, Molecular Device SpectraMax® M5, BMG LABTECH PHERAstar, FLUOstar OPTIMA, BioTek FLx800™, Synergy™ 2, and Synergy™ 4, and ThermoFisher Varioskan. Contact Drug Discovery Technical Support or e-mail us directly at drugdiscovertech@invitrogen.com for instrument-specific setup guidelines.

- Precision pipettes with disposable plastic tips that can accurately deliver volumes of 2–20 µL.
- Ultrapure deionized H_2O.
- Plastic tubes with low protein binding for diluting and aliquoting assay components.
- White (Corning 3574) or Black (Corning 3676) Microtiter plates. Other plates, while not tested, may be suitable.

5. Performing the Assay

Kinase reactions can be run in a 384-well or 96-well plate. The reaction is initiated by addition of the Master Mix to the kinase of interest. Since the assay is a homogeneous, single-step format, no washing steps, stop solutions or additional components are required. The assay is simply conducted at 30°C and fluorescence measurements are recorded using either kinetic readings (*e.g.*, readings every 30 seconds for 60 minutes as the assay progresses) or a single endpoint reading after the assay is complete.

5.1 Preparing the Assay Reagents

Prior to setting up the individual reactions, prepare the following solutions:

1. **Peptide Substrate (10X):** Prepare a 100 µM (10X) peptide stock by adding 2 µL of the 1 mM stock to 18 µL of ultrapure water.
2. **ATP Solution (10X):** Prepare a 10 mM (10X) ATP solution by adding 2 µL of 100 mM ATP to 18 µL ultrapure water.
3. **DTT Solution (10X):** Prepare a 2 mM (10X) DTT solution by adding 2 µL of 1 M DTT to 998 µL ultrapure water.
4. **Kinase Reaction Buffer (1X):** Prepare a 1X Kinase Reaction Buffer solution by adding 0.5 mL of 10X Kinase Reaction Buffer to 4.5 mL of ultrapure water.

5.2 Generating a Kinase Titration

The amount of kinase used in an Omnia® assay is highly dependent on the specific activity of the kinase towards a particular substrate, and should be determined empirically. A kinase titration experiment such as the one described here will allow you to choose the appropriate amount of kinase for your particular application and determine the amount of kinase (*i.e.*, excess kinase) required to phosphorylate the peptide to saturation. The signal obtained from a peptide phosphorylated to saturation can be used as the 100% phosphorylation control for those users who wish to determine the percent phosphorylation that is occurring in their particular application.

Prepare a kinase titration by diluting stock kinase with the 1X kinase buffer prepared above. Each dilution in the series should be at 4X the final concentration of kinase in the reaction. For kinases supplied from Invitrogen, a 1:10 dilution is suggested as a good starting point for the series. A 10-point, two-fold serial dilution will result in a dose response curve spanning 4 logs.

Template 1 — Kinase Titration Assay (20-µL reaction)

Step	Description	Vol. per Reaction
1	To make a Master Mix of everything except active kinase, combine:	
	Kinase Reaction Buffer (10X)	2 µL
	Omnia® Peptide Substrate (10X), prepared above	2 µL
	ATP Solution (10X), prepared above	2 µL
	DTT Solution (10X), prepared above	2 µL
	Ultrapure deionized H_2O	7 µL
	Total Volume	15 µL
2	Incubate the Master Mix for 5 minutes at the reaction temperature (kinase-dependent, typically 30°C).	
3	Warm the assay plate in the plate reader to the reaction temperature (typically 30°C).	
4	Add 5 µL of each concentration of 4X kinase to the appropriate wells.	
5	Aliquot 15 µL of the Master Mix into each tube or well to start the reactions. Mix well.	
6	Incubate at 30°C. During incubation, collect fluorescence intensity readings (λ_{ex} 360/λ_{em} 485) at predetermined intervals (*e.g.*, every 30 seconds for 60 minutes).	
7	Plot Relative Fluorescence Units (RFU) vs. Time. Choose an optimal kinase concentration for use in additional assays.	

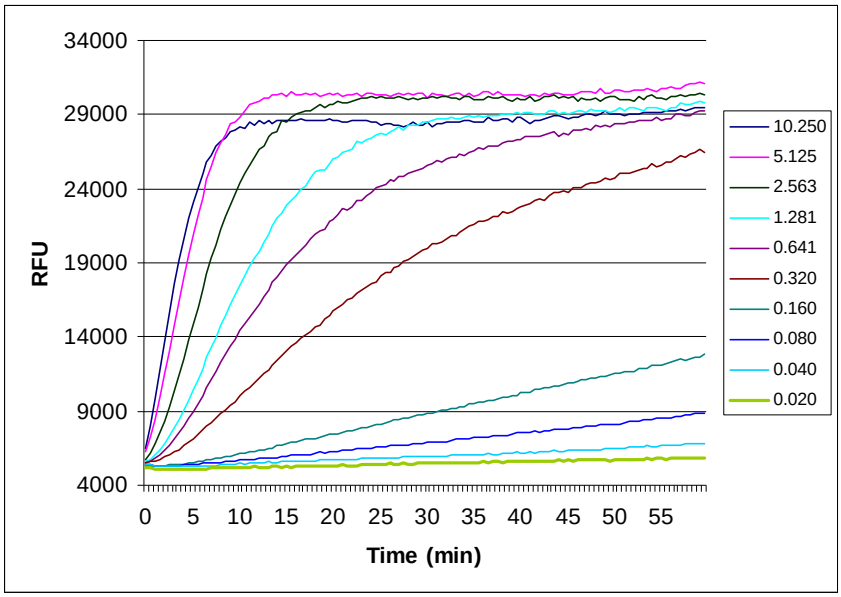


Figure 2. Representative data from the Omnia® Tyr 7 Assay: Syk was serially diluted and then incubated with Omnia® Tyr 7 peptide for 60 minutes at 30˚C. RFU data was collected every 30 seconds and plotted against time. Each line represents a different kinase concentration (µg/mL) as indicated in the legend.

5.3 Kinase Kinetic Activity Analysis

K_m and V_{max} determinations require a kinetic analysis and the generation of a phosphopeptide standard curve. The phosphopeptide standard curve is necessary to convert the RFU signal into a concentration.

To perform the kinetic analysis, combine a fixed concentration of kinase with a serial dilution of peptide substrate. It is important to select a concentration of kinase that will give a linear increase in signal versus time during the initial stages of the kinase reaction. You must empirically determine the exact concentration of kinase required to achieve this linear response via a kinase titration (see **Section 5.2**) and will vary between kinases.

The phosphopeptide standard curve is generated by combining a fixed concentration of kinase with a serial dilution of peptide at the same concentration used in the kinetic assay. Typically the amount of kinase used to generate the phosphopeptide standard curve is higher than the concentration used in the kinetic analysis in order to ensure signal saturation (*i.e.*, 100% phosphorylation of the peptide substrate). The kinase concentration required to reach signal saturation will vary between kinases. A titration of kinase (as outlined in **Section 5.2**) can be performed with the highest concentration of peptide substrate to determine the amount of kinase required to reach saturation.

Peptide substrate preparation: Prepare a two-fold dilution series of substrate using ultrapure water. Each dilution in the series should be at 4X the final concentration of substrate in the reaction. For example, to prepare a dilution with a final substrate concentration of 160 µM, first dilute the 1-mM Omnia® Peptide Substrate to 640 µM (4X) by adding 19.2 µL of Omnia® Peptide Substrate to 10.8 µL of ultrapure water, and then add 5 µL of diluted substrate to the 20 µL reaction as outlined in the following template, for a final concentration of 160 µM.

Reagent preparation: ATP (10X), DTT (10X), and 1X Kinase Reaction Buffer are prepared as described in **Section 5.1**.

Note: K_m determinations frequently require higher substrate concentrations (especially for kinases with K_ms greater than 10 µM).

Template 2 — K_m and V_{max} Determinations (20-µL reaction)

Step	Description	Vol. per Reaction
1	Prepare Peptide Control Master Mix:	
	Kinase Reaction Buffer (10X)	2 µL
	ATP solution (10X)	2 µL
	DTT Solution (10X)	2 µL
	Ultrapure deionized H_2O	9 µL
	Total Volume	15 µL
2	Prepare Phosphopeptide Generation Master Mix:	
	Kinase Reaction Buffer (10X)	2 µL
	Active Kinase (concentration determined to phosphorylate the highest peptide concentration to saturation)	2 µL
	ATP Solution (10X)	2 µL
	DTT Solution (10X)	2 µL
	Ultrapure deionized H_2O	7 µL
	Total Volume	15 µL
3	Prepare Kinetic Reaction Master Mix:	
	Kinase Reaction Buffer (10X)	2 µL
	Active Kinase (concentration determined empirically)	2 µL
	ATP Solution (10X)	2 µL
	DTT Solution (10X)	2 µL
	Ultrapure deionized H_2O	7 µL
	Total Volume	15 µL
4	Incubate the Master Mixes for 5 minutes at the reaction temperature (typically 30°C).	
5	Warm assay plate in the plate reader to reaction temperature (typically 30°C).	
6	Add 5 µL of each serial dilution concentration of Omnia® Peptide Substrate to three sets of wells (one serial dilution for the peptide control, one for the phosphopeptide generation reaction, and one set for the kinetic reaction).	
7	Aliquot 15 µL of the Peptide control Master Mix into one of the peptide serial dilution sets, then aliquot 15 µL of the Phosphopeptide Generation Master Mix into the another peptide serial dilution set, and finally aliquot 15 µL of the Kinase Reaction Master Mix into the final peptide serial dilution set. Mix well.	
8	Incubate at 30°C. During incubation, collect fluorescence intensity readings (λ_{ex} 360/λ_{em} 485) at predetermined intervals (*e.g.,* every 30 seconds for 60 minutes).	

5.4 K_m and V_{max} Data Analysis

Step 1. Subtract background

Subtract the RFU value of the peptide control (*i.e.,* no kinase control) from the RFU value of the kinase reaction for each time point. The RFU value that remains represents the signal from phosphorylated peptide. Since the background fluorescence from the peptide control intensifies at increasing concentrations, the RFU value used for the background subtraction should be from the control peptide at the same concentration as the peptide in the kinase reaction.

Step 2. Determine reaction velocities (v)

Plot the background subtracted RFU values from Step 1 from the kinase reaction versus time and calculate the initial reaction velocities (the slope of the line; RFU/seconds) from the linear portion of the graph.

Step 3. Calculate the slope of the phosphopeptide standard curve

Construct the phosphopeptide standard curve as described in Section 5.3 by graphing the saturating RFU values of each standard curve reaction versus the concentration of peptide substrate in the reaction. Calculate the slope of this standard curve (RFU/µM).

Step 4. Convert reaction velocities to µM/second

Convert the reaction velocities to µM/second by dividing the reaction velocities from Step 2 (RFU/second) by the slope from the phophopeptide standard curve (RFU/µM) from Step 3.

Step 5. Calculate the K_m and V_{max}

Using data analysis software, calculate the K_m and V_{max} by graphing the peptide substrate concentration versus the corrected reaction velocities (µM/second) in a Michaelis-Menten plot and/or prepare a Hanes plot of ([Substrate]/velocity vs. [Substrate]). See the figures below for examples.

K_m and V_{max} Sample Data

A. Michaelis-Menten plot **B. Hanes plot**

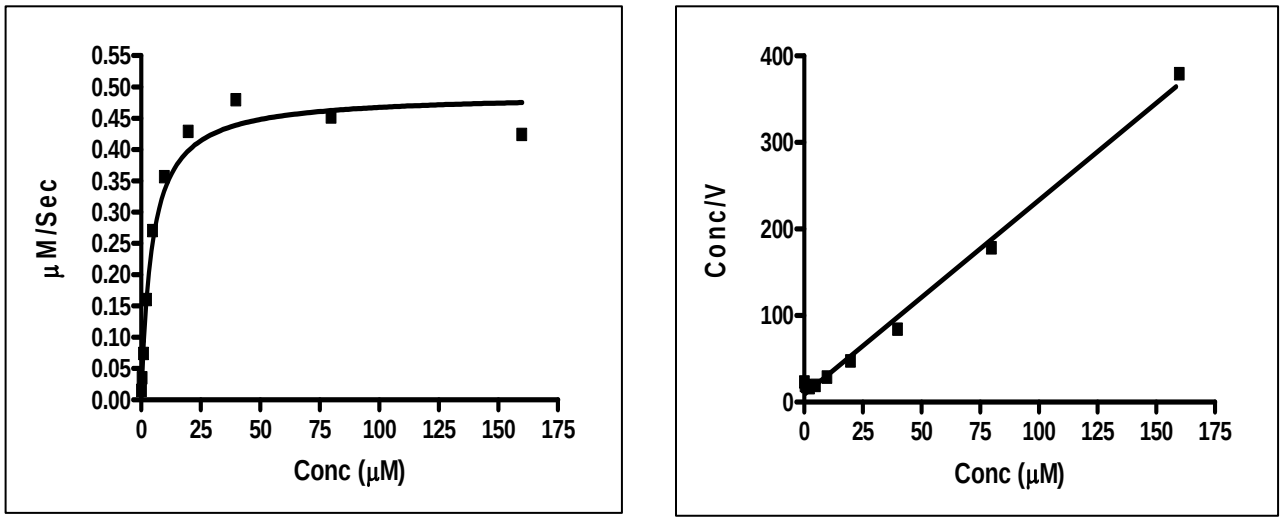


Figure 3. A) Michaelis-Menten plot and B) Hanes plot of experimental results of PKA kinase initial reaction velocities (v) determined at a variety of Omnia ® Ser/Thr Peptide 2 concentrations ([S/T Peptide 2] 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, and 160 µM). The resulting kinetic constants derived from these plots were K_m = 4.5 µM, V_{max} = 1696 nmoles/min/mg. For the Hanes plot, K_m is determined by the negative of the x-intercept ($x = -K_m$ when $y = 0$) of the linear fit of the data. V_{max} is calculated from the y-intercept ($y = K_m/V_{max}$ when $x = 0$) of the same linear fit and the value of K_m.

5.5 Determining Kinase Inhibitor IC_{50} Values

To determine IC_{50} in the Omnia® Assay, plot the initial reaction velocities against a range of inhibitor concentrations.

Note: The compound concentrations should cover at least five orders of magnitude at ½ log intervals.

Note: The inhibitors should be serial diluted in 100% DMSO at 100X and then diluted with 1X Kinase Buffer 1:10 to generate a 10X stock.

Template 3 — Kinase Inhibitor Screening Assay (20-µL reaction)

Step	Description	Vol. per Reaction	Vol. per 100 Reactions
1	Prepare a Master Mix of everything except active kinase by combining:		
	Kinase Reaction Buffer (10X)	2 µL	200 µL
	Omnia® Peptide Substrate (10X); see Section 5.1	2 µL	200 µL
	ATP Solution (10X); see Section 5.1	2 µL	200 µL
	DTT Solution (10X); see Section 5.1	2 µL	200 µL
	Kinase Inhibitor of interest (10X)	2 µL	200 µL
	Ultrapure deionized H_2O	5 µL	500 µL
	Total Volume	15 µL	1500 µL
2	Incubate Master Mix for 5 minutes at the reaction temperature (typically 30°C).		
3	Warm assay plate in the plate reader to reaction temperature (typically 30°C).		
4	Add 5 µL of 4X kinase to each well to be measured.		
5	Aliquot 15 µL of the Master Mix into each well to start the reactions. Mix well.		
6	Incubate at 30°C. During incubation, collect fluorescence intensity readings (λ_{ex} 360/λ_{em} 485) at predetermined intervals (*e.g.,* every 30 seconds for 60 minutes).		
7	Determine initial reaction velocities (v) for each reaction from the slope of a plot of Relative Fluorescence Units (RFU) vs. Time.		
8	Plot velocity vs. [inhibitor] and determine IC_{50}. Fit to a sigmoidal dose response curve to obtain the IC_{50}, as shown in the graph below.		

Syk Inhibition Curve (IC_{50})

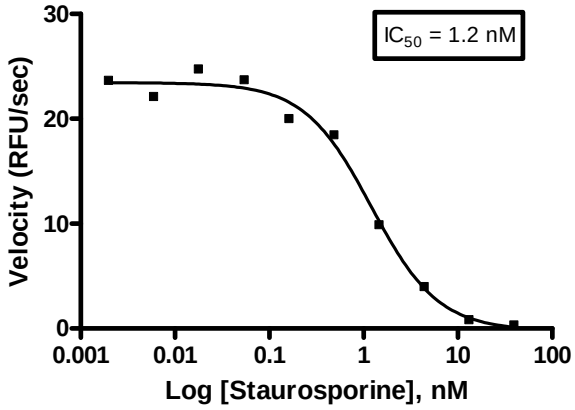


Figure 4: The activity of recombinant Syk was assayed using the Omnia™ Tyr 7 peptide in the presence of increasing concentrations of Staurosporine. The velocity of the reaction (RFUs/sec) was determined and plotted against the Staurosporine concentration.

5.6 Compound Interference

Some samples may contain compounds that interfere with fluorescence and/or activity measurements in this assay. Below is a list of known compounds and their level of interference at specific concentrations:

Compound	Concentration Tested	% Interference
Na_3VO_4	>0.2 mM	50% reduction
NaCl	150 mM	50% reduction

6. References

Shults, M.D. and Imperiali (2003) Versatile fluorescence probes of protein kinase activity. *J. Am. Chem. Soc.* 125(47): 14248-14249.

Shults, M.D., et al. (2005) A multiplexed homogeneous fluorescence-based assay for protein kinase activity in cell lysates. *Nat. Methods* 2:277-283.