KAMIYA BIOMEDICAL COMPANY

PKA (Protein Kinase A) Activity Assay Kit

For the quantitative determination of PKA in cell lysate, tissue extracts and buffer samples

Cat. No. KT-742

For Research Use Only.

PRODUCT INFORMATION
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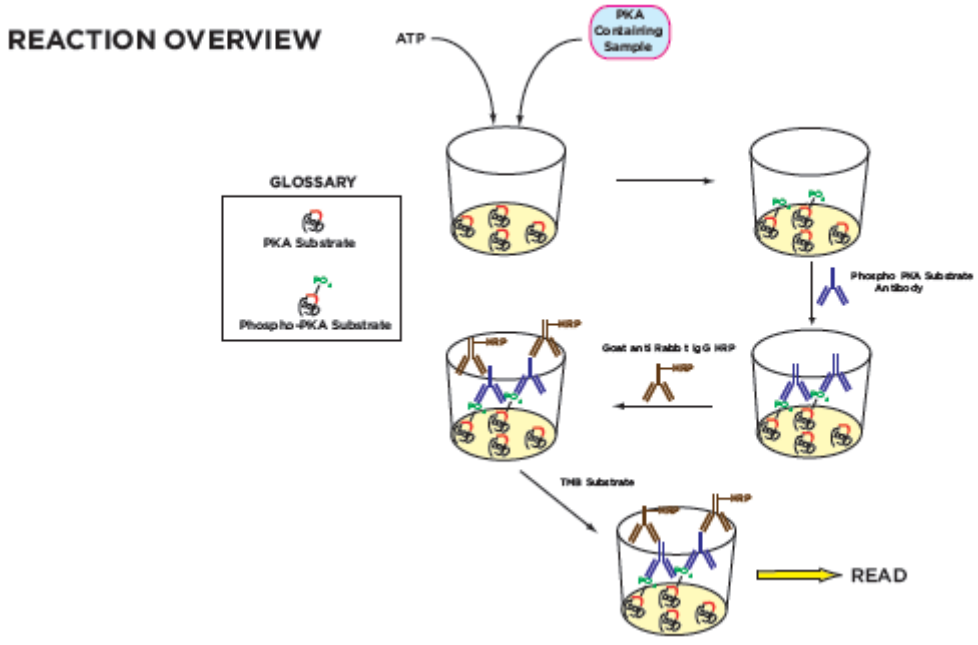
BACKGROUND
PKA was discovered in the laboratory of Edwin G. Krebs in the 1960's. This important class of kinases, refered to as Arg-directed kinases or AGC-family kinases, includes cAMP-dependent protein kinase (PKA or cAPK), cGMP-dependent protein kinase (PKG), protein kinase C, Akt and RSK. These kinases share a substrate specificity characterized by Arg at position 3 relative to the phosphorylated serine or threonine. The second messenger cyclic AMP (cAMP) activates PKA in mammalian cells and controls many cellular mechanisms such as gene transcription, ion transport, and protein phosphorylation. Inactive PKA is a heterotetramer composed of a regulatory subunit (R) dimer and a catalytic subunit (C) dimer. In this inactive state, the pseudosubstrate sequences on the R subunits block the active sites on the C subunits. PKA shares substrate specificity with Akt (PKB) and PKC. Substrates that present this consensus sequence and are phosphorylated by PKA are Bad (Ser^{155}), CREB (Ser^{133}), and GSK-3 (GSK-3α Ser^{21} and GSK-3ß Ser^{9}).

PKA has been implicated in numerous cellular processes, including modulation of other protein kinases, regulation of intracellular calcium concentration, and regulation of transcription. Transcriptional responses to increased cAMP occur through activation of the cAMP response element–binding protein (CREB), cAMP response element modulator (CREM), and activating transcription factor 1 (ATF1). Each of these transcription factors contains a kinase-inducible domain containing a conserved site for phosphorylation by PKA.

PRINCIPLE
The PKA (Protein Kinase A) Activity kit is designed to quantitatively measure PKA activity in a variety of samples. Please read the complete kit insert before performing this assay. A recombinant PKA calibrator is provided to generate a calibration curve for the assay and all samples should be read off the calibration curve. For samples that may have high PKA activity we offer an alternative protocol that reads the substrate reaction at 650 nm. The kit utilizes an immobilized PKA substrate bound to a microtiter plate. Samples containing PKA will, in the presence of the supplied ATP, phosphorylate the immobilized PKA substrate. A rabbit antibody specific for the phospho-PKA substrate binds to the modified immobilized substrate. An antibody specific for rabbit IgG labeled with peroxidase is then added to the plate to bind to the rabbit anti-phospho-PKA substrate. After a short incubation and wash, substrate is added and the intensity of the color developed is directly proportional to the amount of PKA in the samples and calibrators.



COMPONENTS

PKA Substrate 96 Well Plate 1 Plate
Break-apart strip microtiter plate coated with PKA Substrate

PKA Calibrator 2 vials
5,000 Units of recombinant fully active PKA in special stabilizing buffer.
One unit is defined as the amount of PKA required to catalyze the transfer of 1 pmol of ATP phosphate to substrate in 1 minute at 30°C.
PKA Calibrator must be stored at -20°C.

ATP 1 vial
ATP lyophilized. Store in supplied desiccator.

Phospho PKA Substrate Antibody 3 mL
A solution of rabbit antibody specific for phospho-Substrate.

Goat anti-Rabbit IgG HRP Conjugate 3 mL
A solution of goat antibody specific for rabbit IgG labeled with peroxidase.

Kinase Reaction Buffer Concentrate 60 mL
A 2X concentrate containing detergents and stabilizers.

Cell Lysis Buffer 100 mL
A Tris based buffer containing detergents. **Store Frozen** as this buffer contains no preservatives
Cell Lysis Buffer must be stored at -20°C.

Wash Buffer Concentrate 30 mL
A 20X concentrate that should be diluted with deionized or distilled water.

TMB Substrate 11 mL

Stop Solution 5 mL
1M solution of hydrochloric acid. CAUSTIC.

Plate Sealer 2 Each

STORAGE

The unopened kit should be stored at -20°C until the expiration date of the kit.
Once opened the kit can be stored at 4°C up to the expiration date on the kit label, **except for the PKA Calibrator and Cell Lysis Buffer which must be stored at -20°C.** The **Cell Lysis Buffer** has no preservative and must be kept frozen at -20°C. All components of this kit can be stored together at -20°C. The kit must be used prior to the expiration date on the kit box label.

OTHER MATERIALS REQUIRED

Distilled or deionized water.

Glass test tubes.

Shaking plate incubator capable of maintaining 30°C.

Repeater pipet and disposable tips capable of dispensing 10, 25, 50 and 100 µL accurately.

The following Protease inhibitors must**be added to all buffers that are used to measure PKA activity.**
• Phenylmethanesulfonyl fluoride (PMSF), such as Sigma 78830 at 100mM in ethanol.
• A universal protease inhibitor cocktail (PIC) such as Sigma P1860 or Roche 05892970001.

In addition:
• A phosphatase inhibitor, such as Sodium Orthovanadate (**See activation**

instruction opposite.), or a phosphatase inhibitor cocktail, such as Sigma P5726, must added to the Cell Lysis buffer.

Colorimetric 96 well microplate reader capable of reading optical density at 450 and 650 nm.

Software for converting raw relative optical density readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

DualRead System

This kit uses our unique DualRead system. We include instructions for an alternative high calibrator which would typically generate ODs at 450 nm too high to be read on most plate readers. By reading the plate at 650 nm (where TMB optical density is about 3 fold lower) immediately before addition of the Stop Solution some samples outside the normal calibration curve range can be read.

PRECAUTIONS

The PKA Calibrator and Cell Lysis Buffer MUST be stored at -20°C.

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

The coated plate needs to be stored desiccated. The silica gel pack included in the foil ziploc bag will keep the plate dry. The silica gel pack will turn from blue to pink if the ziploc has not been closed properly.

This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturers Wash Buffers, containing sodium azide will inhibit color production from the enzyme. Make sure **all** buffers used for samples are **azide free**. Ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer.

The Stop Solution is acid. The solution should not come in contact with skin or eyes. Take appropriate precautions when handling this reagent.

SAMPLE TYPES

This assay has been validated for Jurkat cell lysates. Samples containing visible particulate should be centrifuged prior to using.

SAMPLE PREPARATION

Cells **must** be lysed in the Activated Cell Lysis Buffer, after addition of protease inhibitors and either **activated orthovanadate** or a phosphatase inhibitor cocktail to the provided Cell Lysis Buffer. All cells and the lysates made from them **must** be stored at ≤ -70°C and should be stored as aliquots for single use. **Do not** freeze-thaw samples. **Do not** store cells or lysates above -70°C.

The preparation of **Activated Sodium Orthovanadate** is as follows:

Preparation of Activated Orthovanadate

200 mM Activated Orthovanadate should be prepared by dissolving 1.84 g of sodium orthovanadate in 45 mL of water. Adjust the pH of the solution to 10 with 1M NaOH or HCl. At pH 10 the solution should be yellow. Boil the solution until it turns **colorless** (approximately 10 min). All of the orthovanadate should dissolve. Cool to room temperature and readjust the pH to 10. Repeat the boiling of the solution and pH readjustment until **the solution is colorless and remains at pH 10**. Adjust the final volume to 50 mL with water. Store the Activated Sodium Orthovanadate in aliquots and freeze at -20°C. Use an aliquot for preparing Activated Cell Lysis Buffer and discard.

Preparation of Activated Cell Lysis Buffer

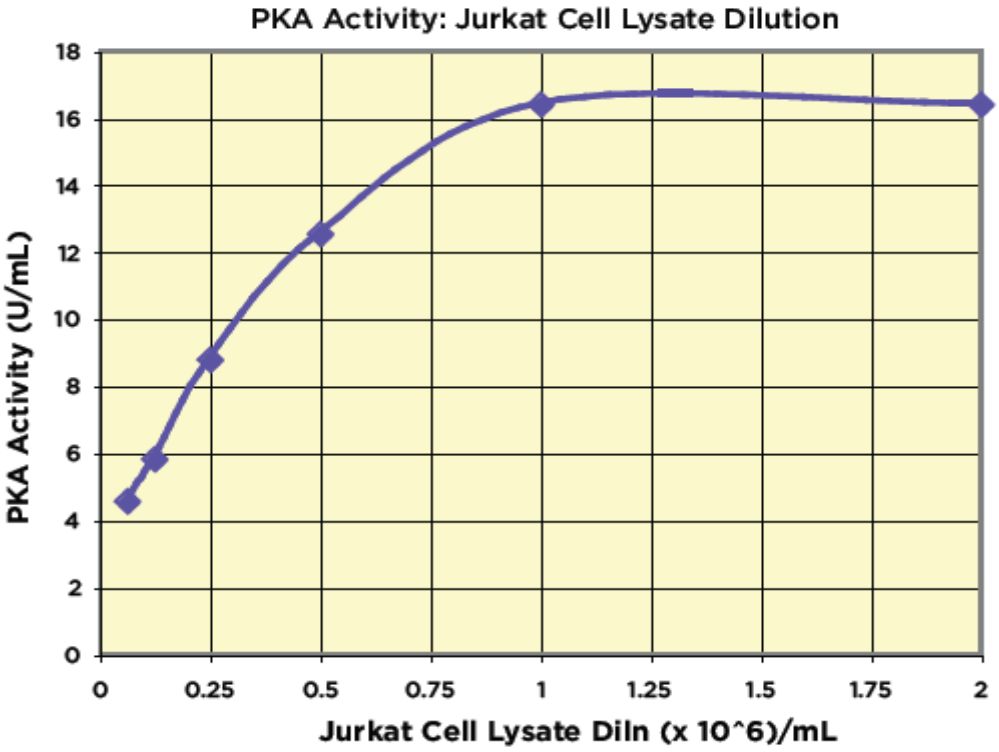
Prepare the Activated Cell Lysis Buffer by addition of 1 µL of PIC per mL of Cell Lysate Buffer. Add 1 mM PMSF and 10 mM **Activated Orthovanadate**. The resulting Activated Cell Lysis Buffer is a pH 8 Tris based buffer containing 1% NP-40 as a cell disruption agent. This assay may not be compatible with other cell lysis buffers containing high concentrations of SDS or other detergents and erroneous activity measurements may result.

Cell Lysis

Add prepared Activated Cell Lysis Buffer to the cells (for Jurkat cells, we lysed at 100 million cells per mL). Incubate for 30 minutes on ice with occasional vortexing. Centrifuge at 10,000 rpm at 4°C for 10 minutes and carefully aspirate off the supernatant for analysis. Supernatants can be frozen at ≤ -70°C for later analysis.

The supernatants should be diluted at least 1:10 into prepared Kinase Assay Buffer prior to running in the assay. **It is recommended that a control lysate be serially diluted in Kinase Assay Buffer to determine the appropriate dilution to obtain a linear response.** See Graph.

Samples diluted in Kinase Assay Buffer can be frozen at ≤ -70°C for analysis later.



REAGENT PREPARATION

Allow the kit reagents to come to room temperature for 30 minutes, **except for the calibrator which must be kept on ice**. We recommend that all calibrators and samples be run in duplicate to allow the end user to accurately determine PKA activity. Keep all samples on ice and ensure they have been diluted appropriately prior to running them in the kit.

Kinase Assay Buffer - **CRITICAL STEP!**
Dilute Kinase Reaction Buffer Concentrate 1:2 by adding one part of the concentrate to one part of deionized water. Add 0.5 µL/mL of PIC and PMSF to 1 mM to make **Kinase Assay Buffer**.
Use within 8 hours.

ATP
Prior to opening vial, tap vial on bench top to ensure contents are in base of vial. Add 1.2 mL of prepared Kinase Assay Buffer to the ATP vial. Vortex to solubilize. Once diluted, store any unused ATP solution at -20°C for up to 3 months.

Wash Buffer
Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted this is stable at room temperature for 3 months.

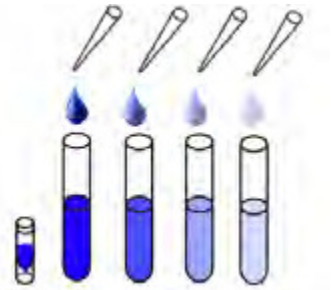
Calibrator Preparation
Spin down the contents of the PKA Calibrator vial in a microcentrifuge for 2 minutes at 14,000 rpm at 4°C. **Keep all calibrators on ice during use.**

Prepare an **Intermediate Stock** dilution by pipetting 1 mL of prepared Kinase Assay Buffer into the PKA calibrator vial. **Invert vial and vortex thoroughly to ensure complete mixing of contents**. This **Intermediate Stock** will have an activity of 5,000 Units/mL.

Label five tubes as #1 through #5. Pipet the calibrators using the **Intermediate Stock** according to the table below. The activity of PKA in tubes 1 through 5 will be 25, 20, 15, 10, and 5 Units/mL.

Alternative High Calibrator For samples that may exceed 25 U/mL prepare the 40 U/mL Alternative Calibrator by pipetting 8 µL of Intermediate Stock into 992 µL of prepared **Kinase Assay Buffer**.

Keep all Calibrators on Ice and use within 30 minutes of preparation.



Standard Activity, U/mL	Std 1 (25 U/mL)	Std 2 (20 U/mL)	Std 3 (15 U/mL)	Std 4 (10 U/mL)	Std 5 (5 U/mL)
KINASE ASSAY BUFFER (µL)	995	60	60	60	60
Addition	5 µL Intermediate Stock	240 µL Std 1	180 µL Std 2	120 µL Std 3	60 µL Std 4

ASSAY PROTOCOL

1. Use the plate layout sheet on the back page of the insert to aid in proper sample and calibrator identification.

2. Pipet 40 µL of **Kinase Assay Buffer** as a Zero Calibrator into duplicate wells in the plate.

3. Pipet 40 µL of samples or calibrators diluted in **Kinase Assay Buffer** into duplicate wells in the plate.

4. Add 10 µL of the reconstituted ATP to each of the wells using a repeater pipet.

5. Seal the plate and incubate at 30°C shaking for 90 minutes.

6. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.

7. Add 25 µL of the Goat anti-rabbit IgG HRP conjugate to each well using a repeater pipet.

8. Add 25 µL of the Rabbit Phospho PKA Substrate antibody to each well using a repeater pipet.

9. Seal the plate and incubate the plate at room temperature for 60 minutes with shaking.

10. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.

11. Add 100 µL of the TMB Substrate Solution to each well, using a repeater pipet.

12. Incubate the plate at room temperature for 30 minutes.

DualRead If the blue substrate color of any of your samples appears darker than the 25 U/mL calibrator we recommend reading the plate at 650 nm, immediately prior to adding stop solution.

13. Add 50 µL of the Stop Solution to each well, using a repeater pipet.

14. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.

15. Use the plate reader's built-in 4PLC software capabilities to calculate PKA activity for each sample.

CALCULATION OF RESULTS

Average the duplicate 450 nm (and optional 650 nm) OD readings for each calibrator and sample. Create a calibration curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean ODs for the zero calibrator. The sample activity obtained should be multiplied by the dilution factor to obtain neat sample values.

TYPICAL DATA (450 NM READ)

Sample	Mean OD (450nm)	Net OD (450nm)	PKA Activity (U/mL)
Standard 1	1.272	1.223	25
Standard 2	0.929	0.880	20
Standard 3	0.618	0.569	15
Standard 4	0.363	0.314	10
Standard 5	0.155	0.106	5
Zero	0.049	0.000	0
Sample 1	0.158	0.109	5.91
Sample 2	1.000	0.951	20.9

TYPICAL DATA (650 NM READ)

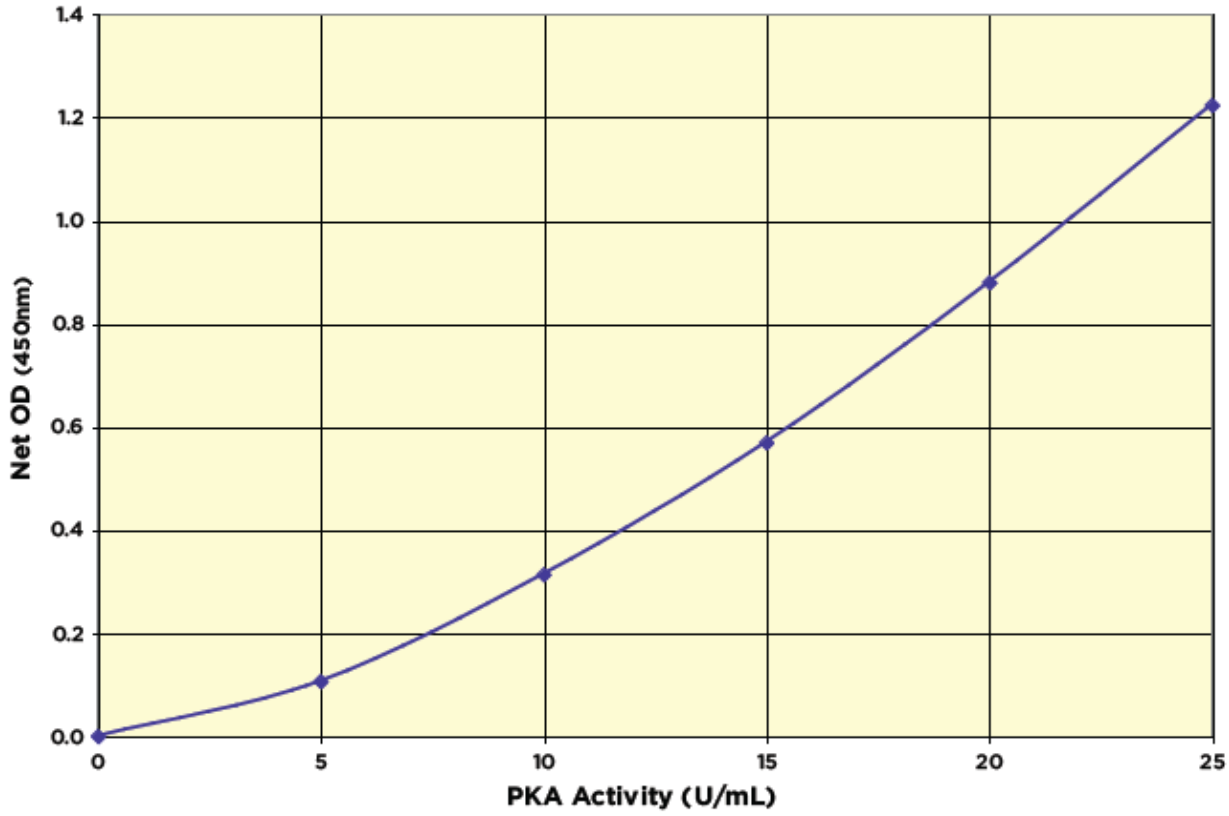
Sample	Mean OD (650nm)	Net OD (650nm)	PKA Activity (U/mL)
Alt. Std.	0.564	0.528	40
Standard 1	0.42	0.384	25
Standard 2	0.307	0.271	20
Standard 3	0.23	0.195	15
Standard 4	0.102	0.066	10
Standard 5	0.06	0.025	5
Zero	0.036	0	0
Sample 1	0.274	0.238	18.8
Sample 2	0.109	0.073	7.86

Always run your own calibration curve for calculation of results. Do not use this data.

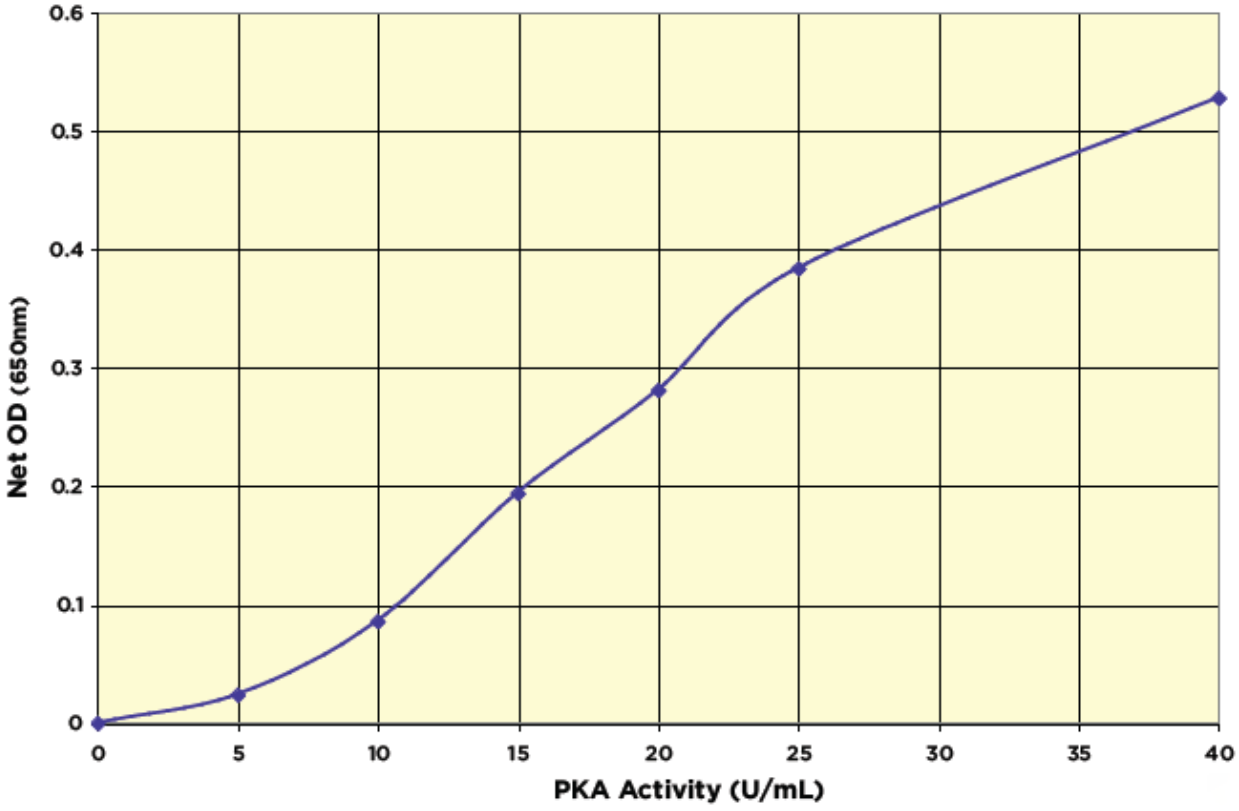
Conversion Factor: 1 ng/mL of human RBP is equivalent to 47.62 pM RBP.

Typical Calibration Curves

450 nm Read



650 nm Read

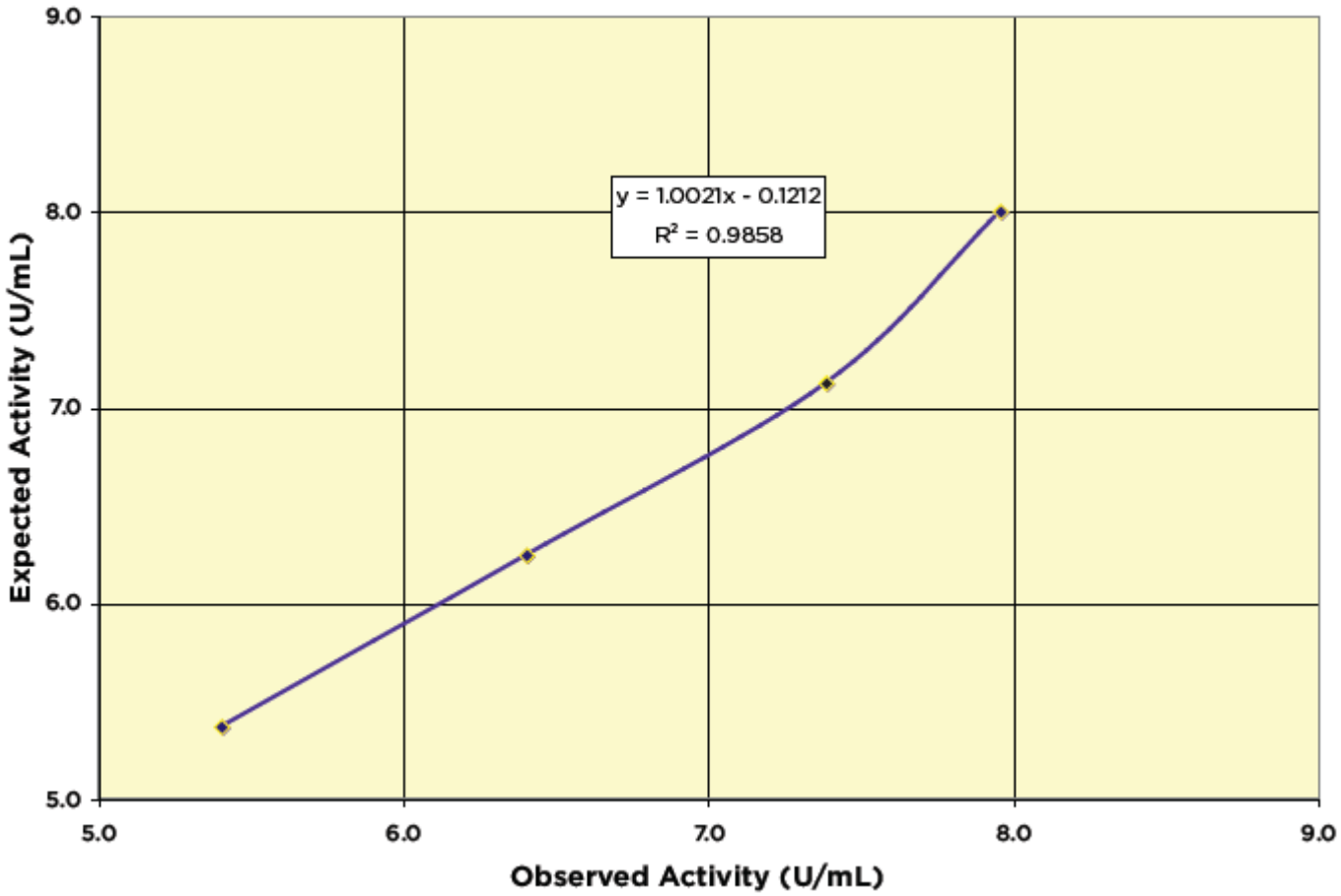


VALIDATION DATA

Sensitivity
Sensitivity was calculated by comparing the OD's for twenty wells run for each of the zero and the 5 Unit/mL calibrator. The detection limit was determined at two (2) standard deviations from the zero along the calibration curve.
Sensitivity was determined as 0.366 Units/mL. This is equivalent to 14.6 milli Units/sample.

Linearity
Linearity was determined by taking two Jurkat cell lysate samples, one with a high PKA activity and one with a lower PKA activity , and mixing in the ratios given below. The measured activities were compared to the expected values based on the ratios used.

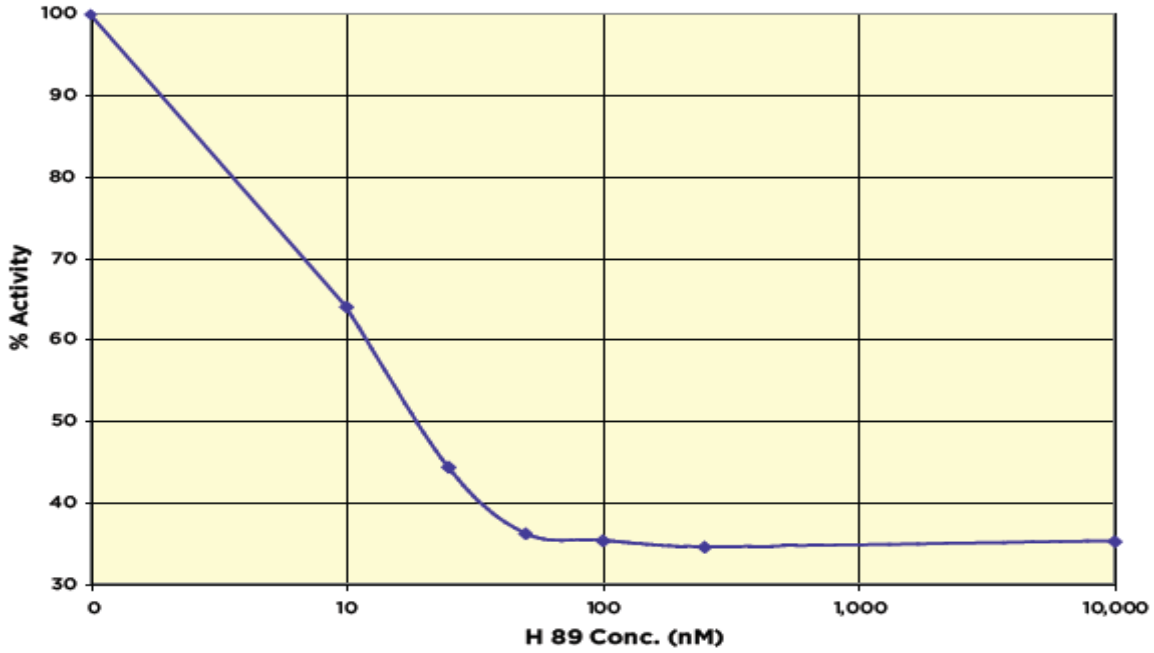
Low Sample	High Sample	Observed Activity (mU/mL)	Expected Activity (mU/mL)	% Recovery
0%	100%	8.88	---	---
20%	80%	7.96	8.00	99.5%
40%	60%	7.39	7.12	103.7%
60%	40%	6.41	6.24	102.6%
80%	20%	5.41	5.37	100.8%
100%	0%	4.49	---	---
			Mean Recovery	**101.6%**



INHIBITION STUDIES

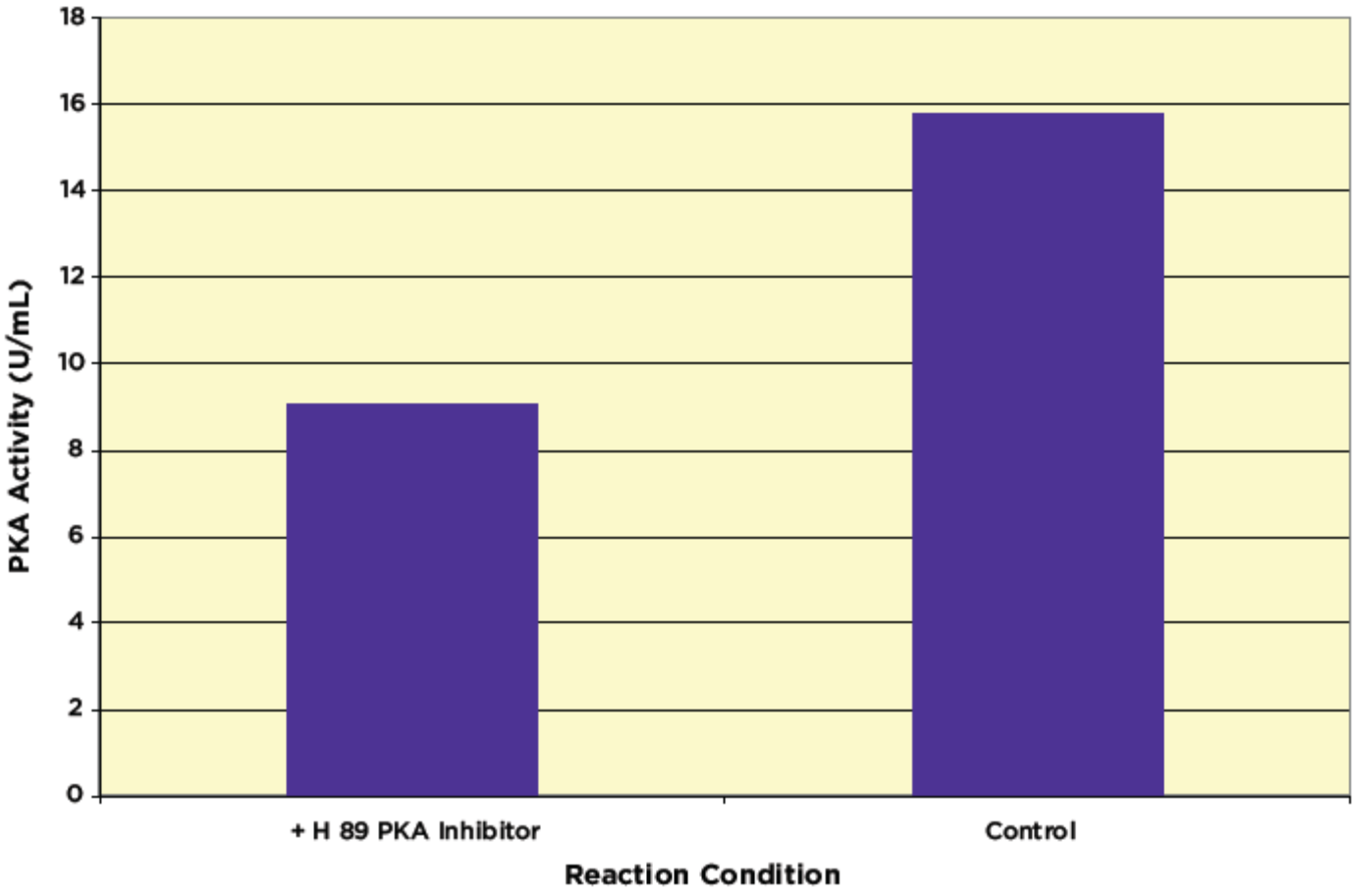
Studies with recombinant PKA

Approximately 30 Units/mL of human recombinant PKA was incubated with H 89 dihydrochloride from 0 to 10,000 nM in Assay Buffer for 30 minutes at room temperature prior to running in the assay. % Activity is expressed by comparison to the activity of the buffer control (28.02 U/mL). 4PLC data comparison determined the IC50% to be 19.1 nM.



Studies with Cell Lysates

Aliquots of a Jurkat cell lysate containing approximately 40,000 cells were treated with the reversible PKA inhibitor H 89 dihydrochloride or Assay Buffer as the control and run in the assay.



INTERFERENTS

A variety of solvents were tested as possible interfering substances in the assay. 0.5% ethanol in the well decreased the activity recorded by 12.7%, whereas 0.10% ethanol in the well decreased activity by 3.7%. 0.5% DMSO in the well decreased activity by 2.8%. 0.1% methanol in the well increased activity by 3.1%. We expect solvent levels at 0.1% of well volume to have little or no effect on the measured activity. A solvent only control should be run by the end user when appropriate.

CROSS REACTIVITY

Protein Kinase	% Cross Reactivity
PKAc alpha	100%
PKAc beta	73%
PKAc gamma	10.1%

FOR RESEARCH USE ONLY

KAMIYA BIOMEDICAL COMPANY
12779 Gateway Drive, Seattle, WA 98168
Tel: (206) 575-8068 Fax: (206) 575-8094
Email: LifeScience@k-assay.com
www.k-assay.com

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