PKA (Protein Kinase A) Colorimetric Activity Kit

Catalog Number EIAPKA (96 tests)

Pub. No. MAN0019010 **Rev** A.0

For safety and biohazard guidelines, see the "Safety" appendix in the *ELISA Technical Guide* (Pub. no. MAN0006706). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Product description

The PKA (Protein Kinase A) Colorimetric Activity Kit is a solid-phase activity assay designed to measure PKA activity in cell lysate, tissue extracts and buffered solutions. The kit uses an immobilized PKA substrate bound to a microtiter plate that is phosphorylated by PKA in the presence of ATP. A two antibody system is then used to detect the presence of the phosphorylated substrate. The assay was characterized with human PKA, but can be used to measure PKA activity in samples from other species.

PKA is a heterotetrameric cAMP-dependent protein kinase, made up of two regulatory subunits and two catalytic subunits in its inactive form. In its active state, the complex dissociates to into a dimeric regulatory subunit, and two enzymatically active catalytic subunits.

Contents and storage

Kit and components are shipped at –20°C. Upon receipt, store the kit at –20°C. Once open, store the kit at 4°C and use within 2 weeks. Store the PKA Standard and Cell Lysis Buffer at –20°C or lower after opening.

Components	Quantity
PKA Standard; 5,000 Units recombinant fully active PKA in a special stabilizing buffer	2 vials
PKA Substrate Plate, 96-well strip-well plate	1 plate
ATP; lyophilized	1 vial
Phospho PKA Substrate Antibody; phospho-substrate specific rabbit antibody	3 mL
Kinase Reaction Buffer Concentrate (2X); containing a reducing agent, detergents, and stabilizers	60 mL
Cell Lysis Buffer; Tris based buffer containing detergents	100 mL
Donkey anti-Rabbit IgG HRP Conjugate	3 mL
Wash Buffer Concentrate (20X)	30 mL
Tetramethylbenzidine (TMB) Substrate	11 mL
Stop Solution; contains 1 M HCl, **CAUSTIC**	5 mL
Plate Sealer	2

Materials required but not supplied

- Distilled or deionized water
- Crushed ice, ice block
- Microtiter plate reader with software capable of measurement at 450 nm and 650 nm
- Calibrated adjustable precision pipettes and glass or plastic tubes for diluting solution
- Shaking plate incubator capable of maintaining 30°C
- 100 mM Phenylmethane sulfonyl fluoride (PMSF) and protease inhibitor cocktail (e.g., Sigma P1860 or Roche 058929700001)
- 200 mM activated sodium orthovanadate or phosphatase inhibitor cocktail (e.g., Sigma P5726)

Procedural guidelines

- Reagents are lot-specific. Do not mix or interchange different reagent lots from various kit lots.
- Solutions containing sodium azide will inhibit the activity of the peroxidase conjugate. Ensure that there is no contamination of labware or the plate washer with azide containing solutions.

Prepare 1X Wash Buffer

1. Dilute 15 mL of Wash Solution Concentrate (20X) with 285 mL of deionized or distilled water. Label as 1X Wash Buffer.
2. Store the concentrate in the refrigerator. 1X Wash Buffer is stable at room temperature for 3 months.

Sample preparation guidelines

- Collect samples in pyrogen/endotoxin-free tubes.
- Freeze samples after collection if samples will not be tested immediately. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well (do not vortex) prior to analysis.
- If large amounts of particulate matter are present in the sample, centrifuge or filter sample prior to analysis.

Prepare Activated Sodium Orthovanadate (200 mM)

1. Dissolve 1.84 g of sodium orthovanadate in 45 mL of water.
2. Adjust solution to pH 10 with 1M NaOH or HCl. At pH 10 the solution should be yellow.
3. Boil the solution approximately 10 minutes until it turns **colorless**. Ensure all of the sodium orthovanadate is dissolved.
4. Cool to solution to room temperature and readjust the pH to 10. Repeat the boiling and pH adjustment step until **the solution is colorless and remains at pH 10**.
5. Adjust the final volume to 50 mL with water.
6. Store the Activated Sodium Orthovanadate in frozen aliquots at –20°C.

Prepare Activated Cell Lysis Buffer

Activated Cell Lysis Buffer is a Tris based, pH 8 buffer containing 1% NP-40 as a cell disruption agent. Using other cell lysis buffers containing high concentrations of SDS or other detergents can result in erroneous activity measurements.

Prepare 1 mL of Activated Cell Lysis Buffer using the following procedure. Scale volumes according to the amount of buffer required.

1. Add of 1 µL of protease inhibitor cocktail per mL of Cell Lysis Buffer.
2. Add 10 µL PMSF (1 mM final concentration) and 50 µL **Activated Sodium Orthovanadate** (10 mM final concentration) per mL of Cell Lysis Buffer. Discard aliquot of thawed Activated Sodium Orthovanadate after use.

Prepare 1X Kinase Reaction Buffer

Note: Keep kinase assay buffer tightly capped and on ice. Use Kinase Assay Buffer within one hour of preparation. Quickly, aliquot remaining 2X Kinase Reaction Buffer into single use aliquots, and freeze at –20°C.

1. When ready to use, thaw the 2X Kinase Reaction Buffer on ice.
2. Dilute the 2X Kinase Reaction Buffer Concentrate 1:2 by adding one part of the concentrate to one part of deionized water.
3. Add 0.5 µL/mL of PIC and PMSF to 1 mM to make 1X Kinase Reaction Buffer. **Use within 1 hour.**

Prepare cell lysate

1. Add Activated Cell Lysis Buffer to cells (e.g., 1×10^6 Jurkat cells) and Incubate for 30 minutes on ice with occasional vortexing.
2. Centrifuge at 10,000 rpm for 10 minutes at 4°C and carefully aspirate off the supernatant for analysis.
3. Supernatants can be frozen as single use aliquots at ≤ –70°C for later analysis. **Do not** freeze-thaw samples.

Dilute samples

Sample activity should be within the range of the standard curve. Because conditions may vary, each investigator should determine the optimal dilution for each application. A control lysate can be serially diluted in 1X Kinase Reaction Buffer to determine the appropriate dilution to obtain a linear response.

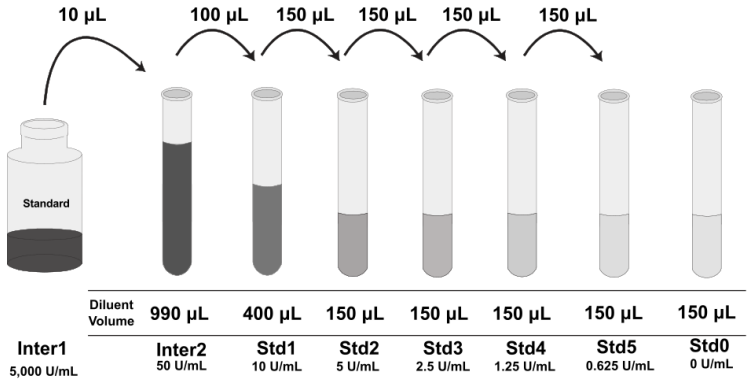
- Perform dilutions in 1X Kinase Reaction Buffer.
- Samples diluted in 1X Kinase Reaction Buffer can be frozen at ≤ –70°C for later analysis.
- Dilute **cell lysate** samples ≥1:15 in 1X Reaction Buffer.

Dilute standards

Note: Use polypropylene tubes for diluting standards.

Note: One unit is defined as the amount of PKA required to catalyze the transfer of 1.0 pmol phosphate from ATP to substrate at 30°C.

1. Centrifuge the vial of standard at 5,000 rpm for 1 minute at 4°C. **Keep all standards on ice during use**.
2. Prepare PKA Standard by adding 1 mL of 1X Kinase Reaction Buffer. Vortex thoroughly. Label as Inter1 (5,000 U/mL) PKA.
3. Add 10 µL Inter1 to 990 µL 1X Kinase Reaction Buffer and label as Inter 2 (50 U/mL) PKA.
4. Add 100 µL Inter 2 to 400 µL 1X Kinase Reaction Buffer. Label as Std1 (10 U/mL) PKA.
5. Add 150 µL 1X Kinase Reaction Buffer to each of 5 tubes labeled as follows: 5, 2.5, 1.25, 0.625, and 0 U/mL PKA.
6. Make serial dilutions of the standard as described below in the dilution diagram. Mix thoroughly between steps.
7. **Use the standards within 30 minutes of preparation**. Discard any remaining unused PKA Standard.



Reconstitute ATP

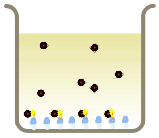
1. Allow the ATP to reach room temperature in the sealed bag before opening.
2. Add 1.2 mL of 1X Kinase Reaction Buffer to the vial of ATP and vortex thoroughly.
3. Store any unused reconstituted ATP at –20°C for up to **3 months**.

Assay procedure

Allow all reagents to reach room temperature before use. Mix all liquid reagents prior to use. **Total assay time is 3 hours.**

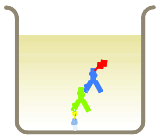
IMPORTANT! Perform a standard curve with each assay.

- Determine the number of 8-well strips required for the assay. Insert the strips in the frames for use. Re-bag any unused strips and frames, and store at 2 to 8°C for future use.



Add sample

a. Add 40 µL of standards or diluted samples (see page 2) to the appropriate wells.
b. Add 10 µL of reconstituted ATP into each well.
c. Seal the plate and incubate for 90 minutes at 30°C with shaking.
d. Aspirate the plate and wash each well 4 times with 300 µL wash buffer.
e. Tap the plate dry on clean absorbent towels.



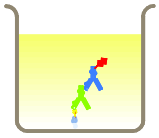
Add detection antibody

a. Add 25 µL of the Donkey anti-Rabbit IgG HRP Conjugate into each well.
b. Add 25 µL of the Phospho PKA Substrate Antibody into each well.
c. Seal the plate and incubate the plate for 60 minutes at room temperature with shaking.
d. Aspirate the plate and wash each well 4 times with 300 µL wash buffer.
e. Tap the plate dry on clean absorbent towels.

Add chromogen

a. Add 100 µL TMB Substrate to each well. The substrate solution will begin to turn blue.
b. Incubate for 30 minutes at room temperature.

Note: TMB should not touch aluminum foil or other metals.



Add stop solution

Add 50 µL Stop Solution to each well. Tap side of the plate gently to mix. The solution in the wells changes from blue to yellow.

Read the plate and generate the standard curve

1. Read the absorbance at 450 nm.
2. Use curve-fitting software to generate the standard curve. A four parameter algorithm provides the best standard curve fit. Optimally, the background absorbance may be subtracted from all data points, including standards, unknowns and controls, prior to plotting.
3. Read the activity of unknown samples and controls from the standard curve. Multiply value(s) obtained for sample(s) by the appropriate factor to correct for the sample dilution.

   Note: Dilute samples producing signals greater than that of the highest standard in 1X Kinase Reaction Buffer and reanalyze. Multiply the activity by the appropriate dilution factor.

Performance characteristics, continued

Standard curve (example)

The following data were obtained for the various standards over the range of 0–10 U/mL (450 nm).

Standard PKA (U/mL)	Optical Density (450 nm)
10	1.639
5	1.056
2.5	0.602
1.25	0.413
0.0625	0.287
0	0.149

Interferents

It is expected that solvent levels at 0.1% of well volume to have little or no effect on the measured activity. Run a solvent only control when appropriate.

A variety of solvents were tested as possible interfering agents in the assay.

- 0.5% ethanol in the well decreased the activity by 12.7%, whereas 0.10% ethanol well decreased activity by 3.7%.
- 0.1% methanol in the well increased activity by 3.1%.
- 0.5% DMSO in the well decreased activity by 2.8%.

Linearity of dilution

Linearity was determined by assaying cell lysate samples with high and low levels of PKA activity mixed in the ratios shown in the following table.

High Sample %	Low Sample %	Expected Conc. (mU/mL)	Observed Conc. (mU/mL)	% Recovery
80	20	8.00	7.96	99.5
60	40	7.12	7.39	103.7
40	60	6.24	6.41	102.6
20	80	5.37	5.41	100.8
			Mean Recovery	**101.6%**

Specificity

The following samples were tested using the assay and cross-reactivity calculated within the standard curve.

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

Sensitivity

The analytical sensitivity of the assay is 0.037 U/mL PKA. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard and the 0.625 U/mL standard were assayed 20 times, and calculating the corresponding concentration.

Limited product warranty

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REF Catalog Number | LOT Batch code | Temperature limitation | Use by | Manufacturer | Consult instructions for use | Caution, consult accompanying documents

Manufacturer's address: Life Technologies Corporation | 7335 Executive Way | Frederick, MD 21704 | USA

The information in this guide is subject to change without notice.

Corporate entity: Bender MedSystems GmbH | Campus Vienna Biocenter 2 | 1030 Vienna, Austria