

ab139460

PDE Activity Assay Kit

(Colorimetric)

Instructions for Use

For the screening of inhibitors and modulators of cyclic nucleotide phosphodiesterase activity.

[View kit datasheet: www.abcam.com/ab139460](http://www.abcam.com/ab139460)

(use www.abcam.cn/ab139460 for China, or www.abcam.co.jp/ab139460 for Japan)

This product is for research use only and is not intended for diagnostic use.

Table of Contents

1.	Principle of the Assay	3
2.	Protocol Summary	5
3.	Materials Supplied	7
4.	Storage and Stability	8
5.	Materials Required, Not Supplied	9
6.	Sample Preparation	10
7.	Assay Protocol	13
8.	Data Analysis	19
9.	Troubleshooting	22

1. Principle of the Assay

Abcam PDE Activity Assay Kit (Colorimetric) (ab139460) is a colorimetric, non-radioactive assay designed in a microplate format. It may be used to screen inhibitors and modulators of cyclic nucleotide phosphodiesterase activity.

The basis for the assay is the cleavage of cAMP or cGMP by a cyclic nucleotide phosphodiesterase. The 5'-nucleotide released is further cleaved into the nucleoside and phosphate by the enzyme 5'-nucleotidase. The phosphate released due to enzymatic cleavage is quantified using Green Assay Reagent in a modified Malachite Green assay. The kit includes Type I cyclic AMP phosphodiesterase (PDE) for validation purposes.

A nonspecific cyclic nucleotide phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) is included as a test control for inhibitor screening. It has an IC_{50} of approximately 25 μ M.

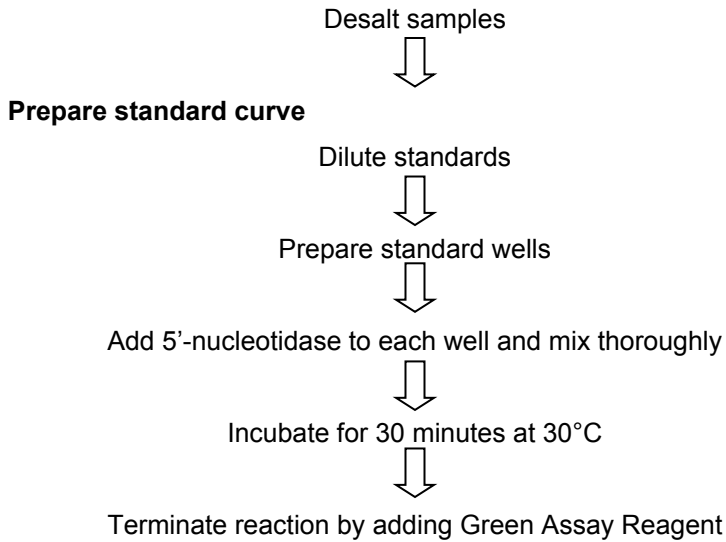
It is important to ensure that compounds active in the cyclic nucleotide phosphodiesterase assay do not inhibit the activity of the 5'-nucleotidase. This can be ascertained by using 5'-AMP rather than 3',5'-cAMP as the substrate. For further information, see section 7.B. "Preparing a Standard Curve".

This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

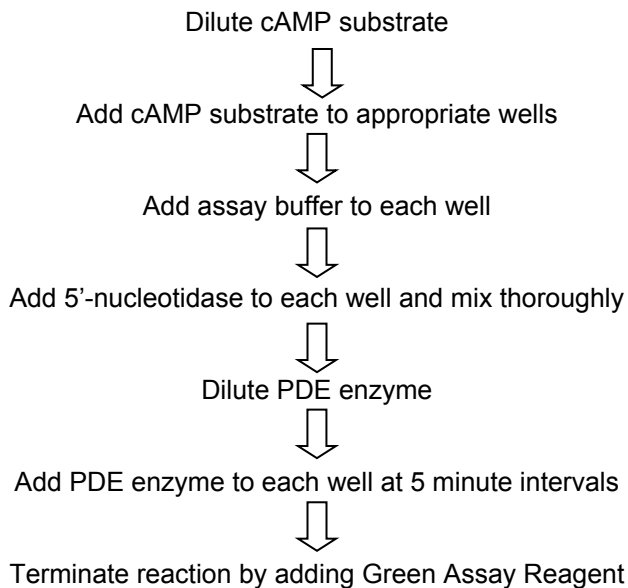
The assay offers the following advantages:

- 1) Non-radioactive
- 2) Convenient one step detection
- 3) Microplate format

2. Protocol Summary

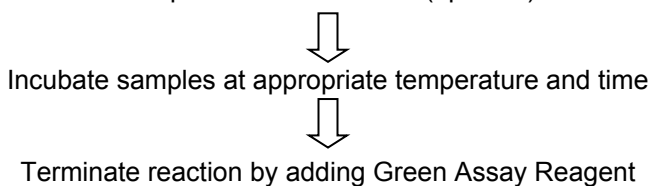


Prepare time course/linearity assay



Prepare test sample/inhibitor assay

Prepare samples containing PDE enzyme, substrate and test compound. Include IBMX (optional)



3. Materials Supplied

Item	Quantity	Storage
PDE Enzyme (from bovine brain) (lyophilized) (4 U per vial)	5 vials	-80°C
5'-Nucleotidase (5 kU/μL) (from <i>Crotalus atrox</i> venom)	1 x 1 mL	-80°C
3',5'-cAMP Substrate (1mM)	1 x 2 mL	-80°C
3',5'-cGMP Substrate (1 mM)	1 x 2 mL	-80°C
PDE Assay Buffer	1 x 40 mL	-80°C
Green Assay Reagent	1 x 20 mL	4°C
5'-AMP Standard (100 μM)	1 x 1 mL	-80°C
5'-GMP Standard (100 μM)	1 x 1 mL	-80°C
PDE Inhibitor (IBMX) (200 μM)	1 x 200 μL	-80°C
Desalting Column	1	4°C
Desalting Resin	1 x 1g	4°C
96-well Clear Microplate (½ Volume)	1	4°C

4. Storage and Stability

- Please note that all components, with the exception of the Green Assay Reagent, Desalting Column, Desalting Resin, PDE Enzyme, and the 5'-nucleotidase can be stored at either -80°C or -20°C.
- **The PDE Enzyme and 5'-Nucleotidase must be stored at -80°C.**
- Because its stability when frozen in solution is poor, the PDE enzyme is provided as 5 lyophilized aliquots. **For highest activity, we recommend that each lyophilized aliquot be dissolved and used for one day's experiments (See Section 7.A. Reagent Preparation).**
- The 5'-nucleotidase is used undiluted at 10 μ L per well. It is stable for up to 6 freeze/thaw cycles (snap freezing with liquid N₂ or dry ice/ethanol), but it may be desirable to make several aliquots if more numerous freeze/thaws are planned.
- One U of 5'-nucleotidase will release 1 pmol phosphate per minute from 200 μ M 5'-AMP, at 30°C in a reaction buffer of 10 mM Tris-HCl, pH 7.4, 0.2 mM MgCl₂.

- One U of PDE enzyme = 1 nmol 3' 5'-cAMP to 5'-AMP per minute under the conditions of the linearity assay
- The Green Assay Reagent is a highly sensitive phosphate detection solution. Free phosphate present on labware and in reagent solutions will greatly increase the background absorbance of the assay. This is detected visually as a change in color from yellow to green. Detergents used to clean labware may contain high levels of phosphate. Use caution by either rinsing labware with dH₂O or employ unused plasticware.

5. Materials Required, Not Supplied

- Microplate reader capable of measuring A_{620} to ≥ 3 -decimal accuracy.
- Pipettes capable of pipetting 10-100 μ L accurately.
- Multi-channel pipetman capable of pipetting 100 μ L (optional).
- Ice bucket to keep reagents cold until use.

6. Sample Preparation

Note: The following procedures are intended only as a guideline. The optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.

Desalting tissue samples by gel filtration

Note: This procedure is intended to remove excess phosphate and nucleotides (which are slowly hydrolyzed to release free phosphate in the presence of the Green Assay Reagent) in the high speed supernatant (HSS) extract.

1. Rehydrate Desalting Resin in a 50 mL conical tube by adding 20 mL of phosphate free dH₂O and vortexing briefly. Allow to set for 4 hours at RT or overnight at 4°C.
2. Decant the dH₂O carefully, then add fresh dH₂O at a 1:1 ratio to the rehydrated resin (~10 mL).
3. Add rehydrated resin to the Desalting Column to obtain a 5 mL settled-bed volume (~5.5 cm bed height). Remove tip from column and allow dH₂O to drain by gravity.
4. Equilibrate column by adding 8 mL of assay buffer and allow to drain by gravity.

5. Place column in a 15 mL centrifuge tube. Centrifuge at 800 x *g* for 3 min at 4°C to displace column buffer. Discard flow-through buffer.
6. Place column in a clean 15 mL centrifuge tube.
7. Add up to 350 µL sample to column.
8. Centrifuge at 800 x *g* for 3 min. Save extract flow-through. This is the desalted cell lysate material to be tested for PDE activity below.
9. Freeze sample immediately at -80°C.

Note: The effective removal of phosphate/nucleotides from the extract should be tested qualitatively by adding 100 µL Green Assay Reagent to 1 µL extract, and a separate sample of 1 µL dH₂O. If no phosphate/nucleotides are present, both samples should remain yellow in color over a time period of 30 min @ RT. The development of a visible green color indicates phosphate contamination, which must be eliminated from the samples before proceeding further.

The rehydrated resin can be stored at 4°C until use. Please note that the volume of Desalting Resin provided in this kit allows user to fill the column twice (5mL settled-bed volume each). In theory, the rehydrated resin can be reused for multiple times (or samples) *. The column will lose efficiency in removing free phosphate gradually after each use. The number of samples that can be desalted by the resin will vary depending on the sample types and amount of phosphate

present in the samples. In general, an average of 5-7 samples can be processed with each 5mL settled-bed volume of rehydrated resin, ie an average of 10-14 samples can be desalted per kit.

*When reusing the rehydrated resin, the column should be rinsed with 4-5 volumes of phosphate-free dH₂O, and then equilibrated with 8 mL of a solution of 10mM Tris-hydrochloric acid, pH 7.4 before loading the next sample.

7. Assay Protocol

A. Reagent Preparation

1. Thaw assay buffer, 5'-nucleotidase, the 3',5'-cAMP substrate, IBMX inhibitor, 5'-AMP and/or 5'-GMP standard. Store all on ice.
2. Prepare 20 U/mL solution of PDE by adding 200 μ L of cold assay buffer to one of the vials of lyophilized enzyme. Store on ice. Note that each of the lyophilized PDE aliquots provided are intended for use in one day's assays only. The enzyme solution may lose substantial activity from freezing/thawing and frozen storage.
3. Warm Green Assay Reagent to room temperature.

B. Preparing a Standard Curve

1. Prepare two dilutions in PDE Assay buffer, 75 μ M and 50 μ M, using either the 5'-AMP or 5'-GMP Standard. For example, bring aliquots of 150 μ L and 100 μ L 5'-AMP Standard (100 μ M) and to 200 μ L with assay buffer.
2. Using PDE Assay Buffer, prepare two sets of 1:1 serial dilutions of the 75 μ M and 50 μ M 5'-AMP standard, plus an assay buffer blank (40 μ L per well). Concentrations of 75, 50, 37.5, 25, 18.75, 12.5, 6.25 μ M correspond to 3, 2, 1.5, 1.0, 0.75, 0.50 and 0.25 nmol 5'-AMP or 5'-GMP (see Table 1):

- a) Add 80 μL of 75 μM 5'-AMP or 5'-GMP standard (Step 1) to well A of assay plate and 80 μL of 50 μM standard to well B.
- b) Add 40 μL 1X assay buffer to wells C through H.
- c) Remove 40 μL from well A and add it to well C. Mix thoroughly by pipetting up and down several times.
- d) Remove 40 μL from well C and add it to well E. Mix well E thoroughly and then remove 40 μL and discard.
- e) Remove 40 μL from well B and add it to well D. Mix thoroughly by pipetting up and down several times. Repeat this process moving and mixing 40 μL from well D to F and then from F to G. Remove and discard 40 μL from well G. DO NOT PROCEED TO THE BLANK WELL 'H'.
- f) Add 10 μL of 5'-nucleotidase (undiluted, 5 $\text{kU}/\mu\text{L}$) to each well and mix thoroughly.
- g) Incubate at 30°C for 30 minutes.
- h) Proceed to "Terminating Reactions" (Section 7.E).

Table 1. Example of standard curve and time course/linearity microplate samples.

Sample Well	5'-AMP/5'-GMP Standard Curve nmol 5'-AMP or 5'-GMP (Columns 1,2)	Time course Min. (Columns 3,4)
A	3.0	90
B	2.0	60
C	1.5	45
D	1.0	30
E	0.75	20
F	0.50	10
G	0.25	5
H	0	0

For highest accuracy, perform all samples in duplicate. See Figures 1 and 2 for example results.

C. Preparing a Time Course/Linearity Assay

1. Dilute cAMP substrate to 0.5 mM with assay buffer.
2. Add 20 μL of cAMP substrate (0.5 mM) to appropriate wells.
The final substrate concentration will be 200 μM .
3. Add 15 μL of assay buffer to each well.
4. Add 10 μL of 5'-nucleotidase (undiluted, 5 kU/ μL) per well.
5. Designate a reaction time to each well (e.g.: 90, 60, 45, 30, 20, 10, 5 and 0 min). See Table 1.
6. Equilibrate microplate to reaction temperature (e.g.: 30°C).
7. Prepare PDE enzyme at 20 U/mL (See Section 7.A. Reagent Preparation). Dilute with assay buffer to 4 U/mL, making enough for the assays planned. Each well will receive 5 μL . Store dilution on ice.
8. Start reactions by addition of 5 μL of PDE enzyme. Total PDE enzyme= 20 mU/well. Make the additions in the reverse time order such that all incubations end at the same time (e.g.: Add 90 min time pt. at $t=0$; add 5 min at $t=85$ min, etc.). The total reaction volume=50 μL .

D. Preparing a Test Sample/Inhibitor Assay

1. Prepare samples containing PDE enzyme, substrate and test compound dissolved in PDE Assay Buffer as listed in Table 2. Include the PDE Inhibitor if desired.
2. Incubate samples at appropriate temperature (e.g. 37°C) and time (e.g.: 60 min).

Table 2: Example of Test Samples/Inhibitor Assay Microplate Samples

	Substrate (0.5 mM)	PDE Assay Buffer	5'-Nase (5 kU/μL)	Test compound	PDE Enzyme (4mU/ μL)
Control	20 μL	15 μL	10 μL	0 μL	5 μL
Test	20 μL	5 μL	10 μL	10 μL	5 μL
PDE Inhibitor	20 μL	5 μL	10 μL	10 μL	5 μL

3. To confirm that an apparent PDE inhibitor does not interfere with the release of phosphate by 5'-nucleotidase, test additional wells using 50 μM 5'-AMP or 5'-GMP standard and the inhibitor(s) in question. Compare the results with the 50 μM standard curve well.

E. Terminating Reactions

1. After incubating wells for desired duration, terminate reactions by addition of 100 μ L Green Assay Reagent. Agitate plate or triturate wells gently to mix.

Note: Avoid production of air bubbles in the wells.

2. Allow color to develop for 20-30 minutes. Be careful to assure samples spend approximately the same time with the reagent before reading on the microplate reader.

3. Read OD_{620nm} on microtiter-plate reader.

Note: Retain microtiter plate for future use of unused wells.

8. Data Analysis

A. 5'-AMP or 5'-GMP Standard Curve

1. Plot standard curve data as OD_{620nm} versus nmol 5'-AMP or 5'-GMP (see Fig. 1).
2. Fit a line to the plotted data using an appropriate linear regression program.
3. Rearrange the equation for best-fit line to solve for nmol of 5'-AMP or 5'-GMP in terms of OD_{620nm}.

$$5'\text{-AMP released} = (\text{OD}_{620\text{nm}} - \text{y-intercept})/\text{slope}$$

SAMPLE CALCULATION:

Best-fit eqn.: $\text{OD}_{620\text{nm}} = 0.232(\text{nmol } 5'\text{-AMP}) + 0.0709$

Rearranged eqn.: $\text{nmol } 5'\text{-AMP} = (\text{OD}_{620\text{nm}} - 0.0709)/0.232$

Example: An unknown produces an $\text{OD}_{620\text{nm}} = 0.400$

$$5'\text{-AMP released} = (0.400 - 0.0709)/0.232 = 1.42 \text{ nmol}$$

4. Substitute OD_{620nm} data obtained from experimental samples (e.g. a PDE reaction) into the rearranged equation to obtain the nmol of 5'-AMP or 5'-GMP produced.

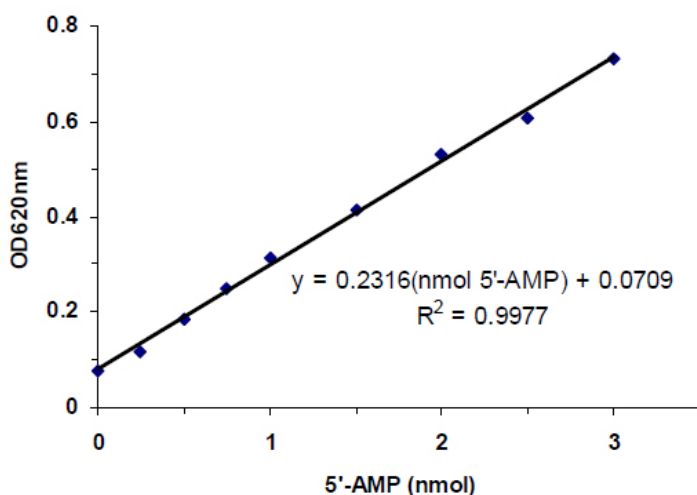


Figure 1. Standard Curve for 5'AMP. Duplicate wells of 5'-AMP dilutions were prepared as described (see Section 8.B). Phosphate was released from 5'-AMP by incubation with 5'-nucleotidase (50 kU/well, 30°C, 30 min.) and the reaction terminated by addition of Green Assay Reagent (100 μ L/well). After 30 min., the phosphate-dependent color reaction was measured by reading OD_{620nm} in a microplate-reading spectrophotometer.

B. Determining the linear range of a PDE reaction time course

Assays for inhibitor screening are most sensitive when the results reflect the initial rate of the enzyme. It is therefore important to choose an incubation time that lies within the initial, linear part of the reaction progress curve. The time courses depicted in Figure 2, remain linear over the course of 45 min. Reasons for the departure from linearity late in a time course may include depletion of substrate, product inhibition and instability of the enzyme. It should also be noted that standard curves for cAMP or cGMP can

themselves become non-linear above 3 nmol ($OD_{620nm} > 0.8$). PDE incubation times that generate more than 3 nmol of cAMP or cGMP should therefore be avoided.

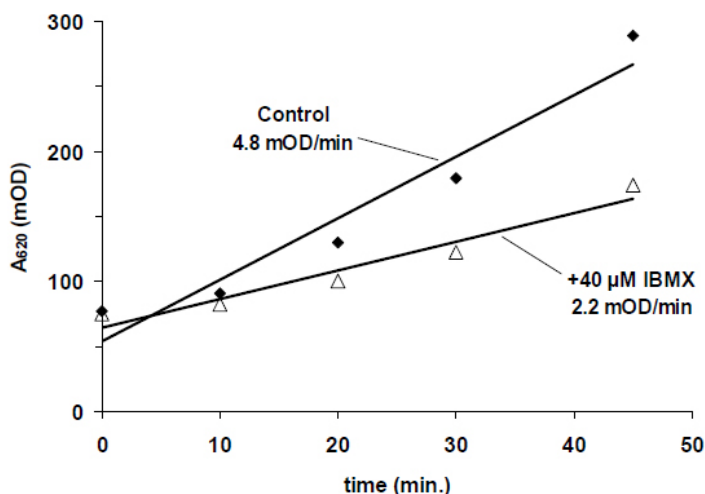


Figure 2. Time Course of cAMP Hydrolysis by PDE, Inhibition by IBMX. PDE enzyme (20 mU/well) was incubated with cAMP (200 μM) and 5'-nucleotidase (50 kU/well) with or without the inhibitor IBMX (40 μM) at 30°C for the indicated times. Reactions were terminated by addition of 100 μL of Green Assay Reagent and OD_{620nm} read 30 min. later. A cAMP standard curve (see Fig. 1) may be used to convert OD_{620nm} data to nmol of 5'-AMP.

9. Troubleshooting

Problem	Reason	Solution
Assay not working	Assay buffer at wrong temperature	Assay buffer must not be chilled – needs to be at RT
	Protocol step missed	Re-read and follow the protocol exactly
	Plate read at incorrect wavelength	Ensure you are using appropriate reader and filter settings
	Unsuitable microtiter plate for assay	Fluorescence: Black plates (clear bottoms); Luminescence: White plates; Colorimetry: Clear plates. If critical, protocol will indicate whether to use flat- or U-shaped wells

Problem	Reason	Solution
Unexpected results	Measured at wrong wavelength	Ensure you are using appropriate reader and filter settings
	Unsuitable sample type	Refer to datasheet for details about incompatible samples
	Sample readings are outside linear range	Concentrate/ dilute samples to be in linear range
Samples with inconsistent readings	Unsuitable sample type	Refer to datasheet for details about incompatible samples
	Too many freeze/thaw cycles	Aliquot samples to reduce the number of freeze/thaw cycles

	Samples are too old or incorrectly stored	Use fresh made samples and store at recommended temperature until use
Problem	Reason	Solution
Lower/higher readings in samples and standards	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Reagents sitting for extended periods on ice	Try to prepare a fresh reaction mix prior to each use
	Incorrect amounts used	Check pipette is calibrated correctly (always use smallest volume pipette that can pipette entire volume)

Problem	Reason	Solution
Standard curve is not linear	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Pipetting errors when setting up the standard curve	Try not to pipette too small volumes
	Incorrect pipetting when setting up the reaction mix	Always prepare a master mix
	Air bubbles in wells	Air bubbles will interfere with readings; try to avoid producing air bubbles and always remove bubbles prior to reading plates

Technical Support

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