CREATIVE BIOMART®
Assay Kit

PDE Activity Colorimetric Assay Kit

Product Information

Cat.No. Kit-0668

Product Overview

PDE Activity Assay Kit (Colorimetric) combines a special dual enzyme system with Green Assay Reagent for phosphate detection to create a unique, non-radioactive, colorimetric assay to detect phosphodiesterase (PDE) activity. This HTS-friendly, mix and read system may be used to screen inhibitors and modulators of cyclic nucleotide phosphodiesterase activity. 96-well microplate format permits rapid assays of large numbers of samples. The basis for the assay is the cleavage of cAMP or cGMP by a cyclic nucleotide phosphodiesterase. The kit includes a Type I PDE as positive control and a non-specific PDE inhibitor, 2-isobutyl-1-methylzanthine (IBMX) as test control for inhibitor screening.

Storage

-80°C

Shipping

Dry Ice

Size

96 tests

Kit Components

3',5'-cAMP Substrate: 1 x 2ml;
3',5'-cGMP Substrate: 1 x 2ml;
5'-AMP standard: 1 x 1ml;
5'-GMP Standard: 1 x 1ml;
5'-Nucleotidase (from Crotalus atrox venom): 1 x 1ml;
96-well Clear Microplate (1/2 Volume): 1 unit;
Desalting Column: 1 unit;
Desalting Resin: 1 x 1g;
Green Assay Reagent: 1 x 20ml;

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PDE Assay Buffer: 1 x 40ml;
PDE Enzyme (from bovine brain): 5 vials*4U;
IBMX (PDE Inhibitor): 1 x 200µl

Materials Required but Not Supplied

Microplate reader capable of measuring A620 to ≥3-decimal accuracy.
Pipet(s) capable of pipetting 5-100 µL accurately
Multi-channel pipet capable of pipetting 100µL (optional).
Ice bucket to keep reagents cold until use.

Assay Protocol

1. 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. NO WARRANTY OR GUARANTEE OF PERFORMANCE USING THESE PROCEDURES IS MADE OR IMPLIED.
2. PRECAUTIONS:
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 diH2O or employ unused plasticware.
To desalt 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 Column 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. After rehydration, store unused resin 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 Chromatography 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.

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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.

TIP: 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!

Preparing assay reagents:

1. Thaw assay buffer, 5'-nucleotidase, the 3', 5'-cAMP substrate, IBMX (PDE Inhibitor), 5'-AMP and/or 5'-GMP standard. Store all on ice.
2. Prepare 20 U/ml solution of PDE by adding 200 µL 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.

Preparing a standard curve:

1. Prepare two dilutions in PDE Assay buffer, 75 µM and 50 µM, using either the 5'-AMP or 5'-GMP Standard. For example, bring aliquots of 150 µL and 100 µL 5'-AMP Standard (100µM) and to 200 µL with assay buffer.
2. Using PDE Assay Buffer, prepare two sets of 1:1 serial dilutions of the 75 µM and 50µM 5'-AMP standard, plus an assay buffer blank (40 µL per well). Concentrations of 75, 50, 37.5, 25, 18.75, 12.5, 6.25 µM 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.

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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.
f) Repeat this process moving and mixing 40 µL from well D to F and then from F to G.
g)Remove and discard 40 µL from well G. DO NOT PROCEED TO THE BLANK WELL 'H'.
h)Add 10 µL of 5'-nucleotidase (undiluted, 5 kU/µL) to each well and mix thoroughly.
i) Incubate at 30°C for 30 minutes. Proceed to section titled "To terminate reactions" .
To prepare a time course/linearity assay:
1. Dilute cAMP substrate to 0.5 mM with assay buffer.
2. Add 20 µL of KI-180 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.: 30, 20, 10, 5 and 0 min). See Table 1.
6. Equilibrate microplate to reaction temperature (e.g.: 30°C).
7. Prepare PDE at 20 U/ml. (See "Preparing assay reagents") Dilute with assay buffer to 4 U/mL, making enough for the assays planned. Each well will receive 5µL. 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 30 min time pt. at t=0; add 5 min at t=25 min, etc.). The total reaction volume= 50 µL.
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	30
B	2.0	20
C	1.5	10

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D 1.0 5
E 0.75 0
F 0.50 -
G 0.25 -
H 0 -
† For highest accuracy, perform all samples in duplicate.
To prepare a test sample/inhibitor assay:
1. Prepare samples containing PDE, substrate and test compound dissolved in assay buffer as listed in Table 2. Include the IBMX (PDE Inhibitor) if desired.
2. Incubate samples at appropriate temperature (e.g.: 30°C) and time (e.g.: 30 min).
3. TABLE 2. EXAMPLE OF TEST SAMPLE/INHIBITOR ASSAY MICROPLATE SAMPLES.
Substrate (0.5 mM) Assay Buffer 5'-Nase. (5 kU/µL) Test cpd PDE (4 mU/µL)
CONTROL 20 µL 15 µL 10 µL 0 µL 5 µL
TEST 20 µL 5 µL 10 µL 10 µL 5 µL
IBMX 20 µL 5 µL 10 µL 10 µL 5 µL
4. 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.
To terminate reactions:
1. After incubating wells for desired duration, terminate reactions by addition of 100 µL 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 OD620nm on microtiter-plate reader.
4. Perform data analysis (see below).
5. NOTE: Retain microtiter plate for future use of unused wells!

Analysis

To calculate results:
1. Plot standard curve data as OD620nm versus nmol 5'-AMP or 5'-GMP.

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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 OD620nm.
5'-AMP released = (OD620nm – y-intercept)/slope
(See SAMPLE CALCULATION below.)
4. Substitute OD620nm 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.
SAMPLE CALCULATION:
Best-fit eqn.: OD620nm = 0.232(nmol 5'-AMP) + 0.0709
Rearranged eqn.: nmol 5'-AMP = (OD620nm -0.0709)/ 0.232
Example: An unknown produces an OD620nm= 0.400
5'-AMP released = (0.400 - 0.0709)/0.232 = 1.42 nmol

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