MA-0160

Phosphodiesterase Activity Assay

(100 wells, Fluorometric, Ex/Em = 370/450 nm, Store at -20°C)

Introduction:

The phosphodiesterase family of enzymes plays a significant role role in metabolism by regulating the levels of cyclic nucleotides, mainly cAMP and cGMP. These cyclic nucleotides are second messengers involved in a host of pathways which control many different physiological processes. PDEs catalyze the hydrolysis of cAMP and cGMP, inactivating them, leading to the termination of downstream signaling pathways. The influence of the PDE family is extensive and diverse. By controlling the levels of cAMP and cGMP, PDEs impact cell growth, differentiation, contractility, neurotransmission, and immune response. PDE Dysfunction has been linked to various diseases, including cardiovascular and neurological conditions, and cancer. PDE inhibitors have emerged as essential therapeutic agents to modulate cyclic nucleotide signaling in these diseases. The specificity and tissue distribution of the PDEs permits much flexibility in their inhibition. Different PDE isoforms exist in different cell types. PDE3 is found mainly in cardiac and vascular tissue, PDE 5 in smooth muscle cells. There are more than 20 isoforms of PDE4 alone. Understanding PDE function is important in many areas of biochemistry and medicine. AkrivisBio's Phosphodiesterase Activity assay is a simple, sensitive means of measuring PDE activity in a wide range of biological samples with a sensitivity into the low picomole/minute (µU) range.

Assay Principle:

PDE activity hydrolyzes a synthetic fluorescently quenched fret substrate.
The cleaved product fluoresces intensely (Excitation 370 nm; Emission 450 nm)

Assay Components:

PDE Assay Buffer	25 ml	WM	MA-0160A
PDE Substrate	15 µl	Red	MA-0160B
Coumarin Standard (50 µM)	100 µl	Yellow	MA-0160C
PDE Positive Control	lyoph	Purple	MA-0160D

Storage Conditions and Handling Considerations:

Store the unopened kit at -20°C. Centrifuge the vials for a few seconds before opening. Bring all components to room temperature before using them.
PDE Assay Buffer: Warm to room temperature before use.
PDE Substrate: Store at -20ºC. Add 135 µl DMSO to the vial before use and mix well. Aliquot into convenient portions and store at -20ºC.
Coumarin Standard: Store at -20ºC. Vortex well before use.
PDE Positive Control: Positive control is stable at -20°C for 1 year in the lyophilized state. Reconstitute with 22 µl Assay Buffer. There is sufficient material for 5 – 10 Positive Control wells. Aliquot to 4-5 µl portions and store at -80°C. Reconstituted positive control should last 2 months.

Phosphodiesterase Activity Assay Protocol:

1. Prepare the plate reader for data acquisition in kinetic mode so the prepared plate below can be immediately read.

2. Standard Curve: Dilute the Coumarin Standard 1:20 by adding 5 µl to 245 µl of DMSO (gives a 1.0 µM solution)**.** Mix well and transfer 0 – 5 – 10 – 15 – 20 – 25 µl to a series of wells in a white 96 well plate. Bring the well volumes to 100 µl with Assay Buffer giving0 – 5 – 10 – 15 – 20 and 25 pmoles per well respectively of the Coumarin standard. Mix well by pipetting. Verify the absence of bubbles in the wells.

3. Sample Preparation: Homogenize cells (1 x 10^6) with 100 µl ice cold Assay Buffer. Place on ice for 10 minutes then centrifuge at 16,000 x g for 10 minutes at 4°C and transfer the clear supernatant to a fresh tube. You can optionally determine protein concentration on a sample at this point. Protein concentration should be between 5-20 mg/ml. Dilute more concentrated samples with Assay Buffer. Use immediately or store lysates at -80°C.immediately. 5-20 µl of liquid biological samples can be used directly. Samples should be transferred to the plate in duplicate with one of the pair being used as a background control. Adjust all sample well volumes to 50 µl with Assay Buffer.

4. Positive Control: Add 1 - 4 µl of PDE Positive Control into a desired well and adjust the final volume to 50 µl with PDE Assay Buffer. As a background control, add 50 µl of Assay Buffer to a well.

Note: All sample readings should be within the range of the Standard Curve. If any exceed this range, dilute them appropriately and rerun.

5. Initiate Reaction: Each Sample, Positive Control and Background Control well will require 50 µl of Reaction Mix. Prepare sufficient material for the total number of wells to be run containing the following:

	Reaction Mix	Background Control Mix
Assay Buffer	48.5 µl	50 µl
PDE Substrate	1.5 µl	-----

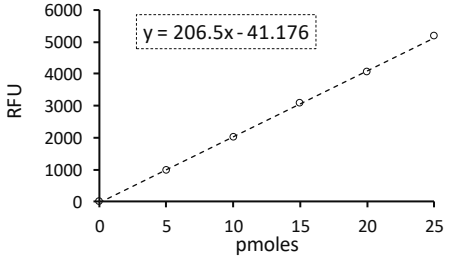
Note: Turbidity upon addition of PDE Substrate to Assay buffer is normal. Just vortex to have it disappear.

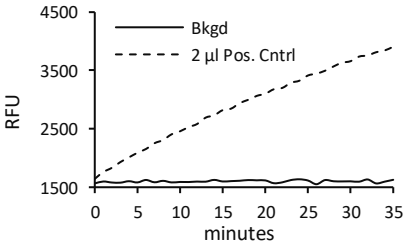
Add 50 µl of Background Control Mix to the Background Control wells first, then add 50 µl of Reaction Mix to the Samples and Positive Control wells.

6. Measurement: Place the plate in the plate reader and immediately start recording fluorescence for 30 - 60 minutes at room temperature. Reaction time depends only upon the amount of PDE activity. The slowest samples will dictate how long to run the data acquisition.

7. Typical Data:

pmole Standard	Standard Raw Values RFU	Background Corrected Values RFU
0	1600.3	0
5	2566.3	966
10	3598.2	1998
15	4668.0	3068
20	5639.5	4039
25	6769.9	5170





8. Calculation: Subtract the zero standard reading from all other standard readings. Plot the Standard Curve. Determine the slope of the Standard Curve (RFU/pmole). Subtract the background control well readings from the paired sample readings. Determine the slope of the background corrected sample enzyme activities (RFU/minute). Divide the slope of each enzyme activity slope by the slope of the Standard Curve (RFU/min)/(RFU/pmole) to convert the enzyme activity to pmole/minute in the well. To convert back to activity in the original sample:

A. Divide the activity value for the well by the volume of sample added to the well = activity per µl of sample (pmol/minute/µl or µU/µl)
B. Multiply the activity per µl of sample by the total volume of sample (supernatant) in step 3 above = total µU per sample
C. Divide total µU per sample by the mg of tissue or # of cells used to make the sample = µU per mg tissue (or per # of cells, etc.)

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