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Document # Inst-LT07-600 03/17

PDELight™ HTS cAMP phosphodiesterase Kit

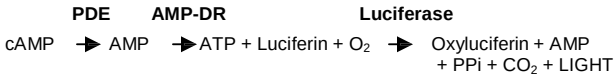
Safety

THESE PRODUCTS ARE FOR RESEARCH USE ONLY. Not approved for human or veterinary use, for application to humans or animals, or for use in clinical or *in vitro* procedures.

Description

Phosphodiesterases (PDEs) function in conjunction with adenylate cyclase to regulate the amplitude of the ubiquitous second messenger signalling molecule, cyclic adenosine monophosphate (cAMP). Phosphodiesterases hydrolyse cAMP to adenosine monophosphate (AMP).

The PDELight™ assay is a non-radioactive, bioluminescent detection system for the activity of phosphodiesterases which utilise cAMP as their preferential second messenger. The AMP produced from the hydrolysis of cAMP is quantified using the PDELight™ AMP detection reagent which converts this AMP directly to ATP. The assay uses luciferase, which catalyses the formation of light from the newly formed ATP and luciferin following the reaction schematic according to the following reaction:



The emitted light is directly proportional to the level of AMP present in the reaction (Fig. 1).

The assay can be performed with any cAMP dependant phosphodiesterase and unlike other assays does not require radioactive substrates, beads, modified substrates or antibodies. The assay can be performed in 96 or 384 well formats and is suitable for miniaturization to 1536 well plates or beyond.

The assay can be easily and rapidly optimized for each phosphodiesterase, with linearity over 20µM AMP, a half life in excess of 2 hours (Fig. 2) and excellent Z' values (Fig. 7). It easily detects known phosphodiesterase inhibitors (Fig. 5) and provides an easily determined end point for IC_{50} analysis (Fig. 6).

Figure 1. Light output correlates directly to AMP concentration with no interference of cAMP

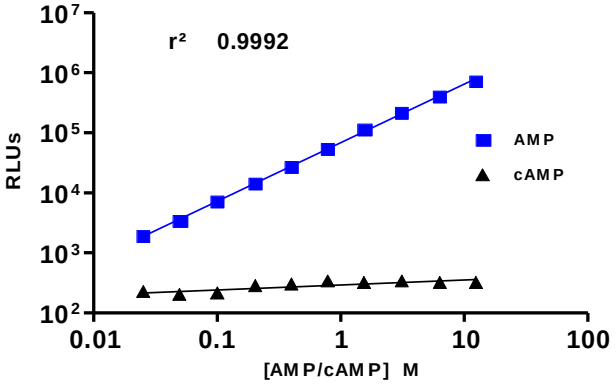


Figure 2. Signal half life

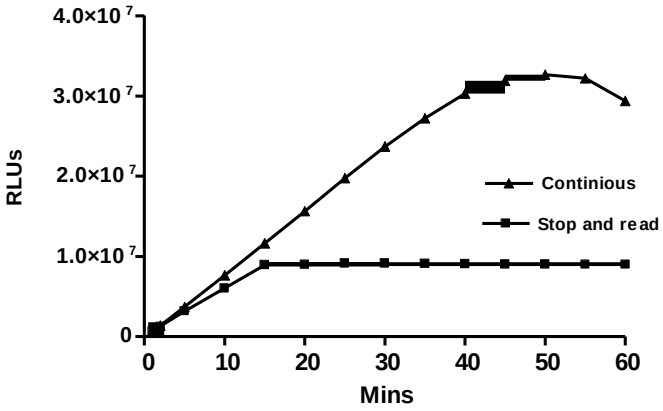


Figure 2. Comparisons of the PDELight™ assay run using the Kinetic (protocol D) and Stop and read (protocol C) methodologies. When the stop solution is added after 10 minutes incubation of the PDE an extremely stable signal is generated. The signal half life of the assay is in excess of 2 hours. Using the Kinetic method the signal is linear for over 30 minutes until the cAMP concentration becomes limiting.

Kit Contents	Size	Cat No.
PDELight™ HTS cAMP phosphodiesterase kit	**500 test**	**LT07-600**

Component	Size	Cat No.
PDELight™AMP detection reagent	10 ml	LT27-250
PDELight™ stop solution	10 ml	LT27-253
PDELight™ reconstitution buffer	20 ml	LT27-256

Kit components should be stored at 2℃-8℃. See kit label for expiry date of the whole kit. See bottle labels for expiry dates of individual components.

NOTE: PDELight™ reagents are available in bulk quantities based upon individual requirements. Your Lonza representative can advise you on the most suitable options.

Equipment and reagents

To be supplied by user:

1. Phosphodiesterase.
2. cAMP.
3. Phosphodiesterase reaction buffer.

The PDELight™ kit requires the use of a luminometer or beta counter. The parameters of the luminometer should be assessed and the conditions below used to produce the correct programming of the machine. If the luminometer has temperature control this should be set to 22℃, the optimal temperature for luciferase.

Microplate Luminometer:

Read time: 0.1 second (integrated).

Beta counters:

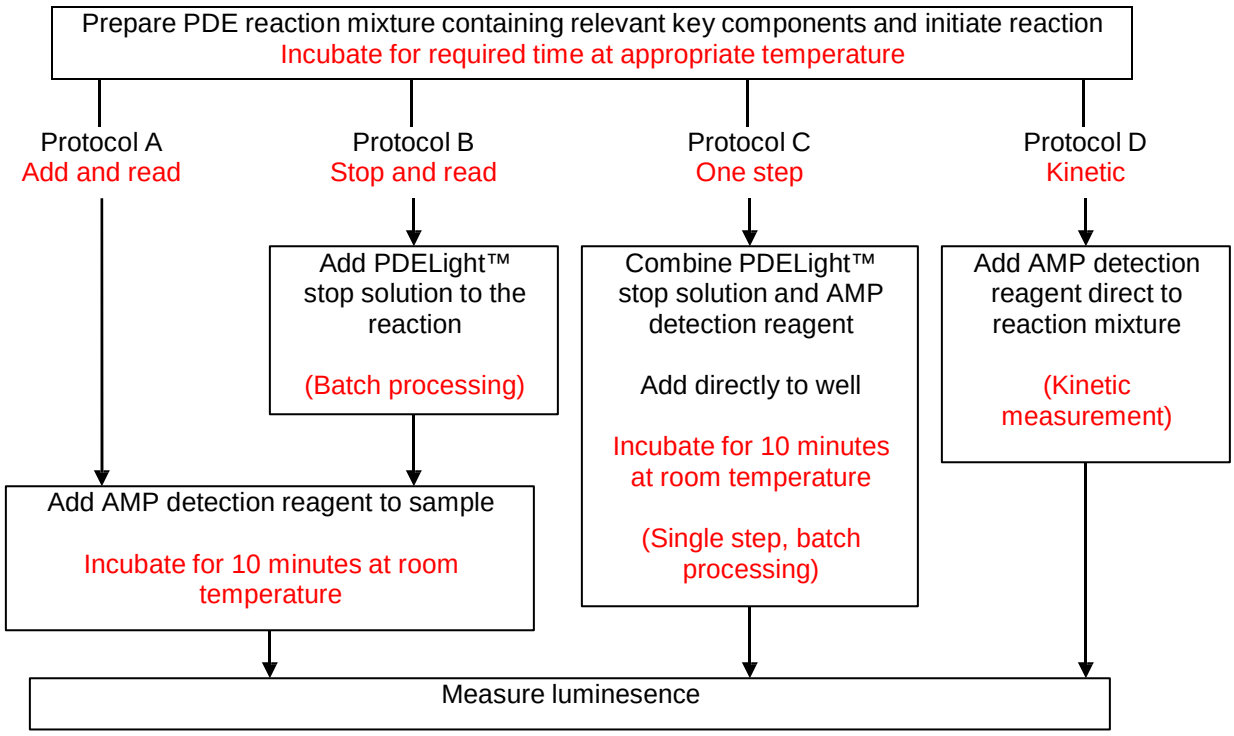
Mode: out of coincidence or luminescence.

Read time: 0.1 second (integrated).

NOTE: The integrated read time of 0.1 second as suggested above is recommended. Integral read times can be adjusted but should be kept between 0.1 to 1 second.

Protocol selection

Figure 3. Flow chart of PDELight™ HTS cAMP phosphodiesterase assay protocols.



Additional equipment and consumables

1. 10 ml sterile pipettes.
2. Opaque white wall microtitre plates suitable for luminescence measurements. The same microplates should be used with beta counters.
3. Multichannel micropipettes – 5 to 50 µl or a suitable micro-dispensing system.

Reagent reconstitution and storage

See Figure 3 for selection of appropriate protocol.

Please read this section carefully to ensure optimal performance for your assay. This procedure requires at least 15 minutes equilibration time.

Protocols A / B and D reconstitution instructions.

(Add and read / Stop and read / Kinetic protocols only)

NOTE: See right column for the Protocol C reconstitution instructions.

AMP detection reagent (lyophilised)

1. Add 10 ml of the reconstitution buffer into the vial containing the lyophilised AMP detection reagent.
2. Replace the screw cap and mix gently. Do not vortex
3. Allow the reagent to equilibrate for 15 minutes at room temperature.

Use reconstituted AMP detection reagent within 6 hours or 24 hours if stored at 4 ^{o}C.

Prolonged storage

Unused reagent can be aliquoted into polypropylene tubes and stored at -20^{o}C or below for up to 1 month protected from light. Allow to equilibrate to room temperature without the aid of artificial heat before use. Once thawed, reagent must not be refrozen.

Stop solution (lyophilised)

NOTE: Not required for Protocols A or D.

1. Add 10 ml of the reconstitution buffer into the vial containing the lyophilised stop solution.
2. Replace the screw cap and mix gently.
3. Allow the reagent to equilibrate for 15 minutes at room temperature.

Use reconstituted Stop Solution within 6 hours or 24 hours if stored at 4℃.

Prolonged storage

Unused reagent can be dispensed into single-use aliquots in polypropylene tubes and stored at -20℃ or below for up to 1 month protected from light. Allow to equilibrate to room temperature without the aid of artificial heat before use. Once thawed, reagent must not be refrozen.

Reconstitution buffer

This is provided ready for use. Store at 2℃-8℃ when not in use.

Protocol C reconstitution instructions

(One step protocol only)

Combined stop solution and AMP detection reagent.

1. Add 10 ml of the reconstitution buffer into the vial containing the lyophilised stop solution.
2. Replace the screw cap and mix gently until all of the lyophilised solid goes into solution. (Do not vortex).
3. Equilibrate at room temperature for 5 minutes.
4. Pour the stop solution into the lyophilised AMP detection reagent.
5. Replace the screw cap and mix gently until all of the lyophilised solid goes into solution. (Do not vortex).
6. Allow the reagent to equilibrate for 15 minutes at room temperature.

Use reconstituted stop solution within 6 hours or 24 hours if stored at 4℃.

Prolonged storage.

Unused reagent can be dispensed into single-use aliquots in polypropylene tubes and stored at -20℃ or below for up to 1 month protected from light. Allow to equilibrate to room temperature without the aid of artificial heat before use. Once thawed, reagent must not be refrozen.

Protocols

NOTE: 96 and 384 well plate formats only - for information on use with 1536 well plate formats contact Scientific Support.

Please ensure you have read the appropriate reconstitution instructions before starting.

See Figure 3 for selection of appropriate protocol.

Protocol A

(Add and Read Protocol)

1. Reconstitute reagents and allow to equilibrate to room temperature.*
2. Prepare phosphodiesterase reaction mixture. (See Trouble Shooting / Main Considerations pg 7).
3. Incubate phosphodiesterase reaction for required time.
4. Add 20 µl of the reconstituted AMP detection reagent to the wells and incubate for 10 minutes at room temperature.
5. Read luminescence using a 0.1 second integration time.

Protocol B

(Stop and read protocol)

1. Reconstitute reagents and allow to equilibrate to room temperature.*
2. Prepare phosphodiesterase reaction mixture. (See Trouble Shooting / Main Considerations pg 7).
3. Incubate phosphodiesterase reaction for required time.
4. Add 10 µl of the Stop Solution to the reaction to stop further phosphodiesterase activity.
5. Add 20 µl of the reconstituted AMP Detection Reagent to the wells and incubate for 10 minutes at room temperature.
6. Read luminescence using a 0.1 second integration time.

Protocol C

(One step protocol)

1. Reconstitute reagents and allow to equilibrate to room temperature.* (See "Protocol C reconstitution instructions" pg 3).
2. Prepare phosphodiesterase reaction mixture. (See Trouble shooting / main considerations).
3. Incubate phosphodiesterase reaction for required time.
4. Combine the stop solution with the AMP detection reagent as described on page 4.
5. Add 20 µl of the combined reconstituted Stop Solution / AMP detection reagent to the wells and incubate for 10 minutes at room temperature.
6. Read luminescence using a 0.1 second integration time.

Protocol D

(Kinetic protocol)

1. Reconstitute reagents and allow to equilibrate to room temperature.*
2. Prepare phosphodiesterase reaction mixture without the cAMP. (See Trouble Shooting / Main Considerations pg 7).
3. Add 20 µl AMP Detection Reagent direct to the phosphodiesterase reaction mixture.
4. Add the required amount of cAMP to start the reaction.
5. Read Kinetics using a 0.1 second integration time.

* Please be aware this will take at least 15 minutes.

Optimising phosphodiesterase reaction conditions

It is essential to optimise the phosphodiesterase reaction conditions with respect to the concentration of cAMP and the phosphodiesterase, in order to get the best performance when using the PDELight™ Reagents.

For both 96 and 384 well formats we recommend a 40 µl reaction volume, 10 µl stop solution (optional) and 20 µl AMP detection reagent. Other volumes may be used providing the ratios are maintained throughout.

NOTE: All dilutions are made using a suitable phosphodiesterase reaction buffer.

Determining optimal cAMP concentration

1. Perform a two fold dilution series of cAMP across the plate using as much phosphodiesterase as practical. (i.e. 20-0 µM cAMP) in a total reaction volume of 40 µl.
2. For the control make the same dilution series, minus the phosphodiesterase.
3. Incubate at room temperature to allow the phosphodiesterase time to consume an adequate amount of cAMP, i.e. 30 – 60 minutes.
4. Add 10 µl of stop solution (optional).
5. Add 20 µl of AMP detection reagent.
6. Incubate for at least 10 minutes at room temperature.
7. Measure luminescence using a 0.1 second integration time.

The optimal cAMP concentration will result in the largest change in luminescence in the completed phosphodiesterase reaction wells compared to that of the control wells. The assay is linear up to 20 µM AMP

Determining optimal phosphodiesterase concentration

1. Perform a two fold dilution series of the phosphodiesterase across the plate, starting with the largest practical amount using the optimal cAMP concentration previously determined in a total reaction volume of 40 µl.
2. Incubate at room temperature for desired time, i.e. 30-60 minutes.
3. Add 10 µl Stop Solution (optional).
4. Add 20 µl AMP Detection Reagent.
5. Incubate for at least 10 minutes at room temperature.
6. Measure luminescence at 0.1 second integration time.

The EC_{50} will lie in the linear range of the phosphodiesterase titration curve. This will be the optimal concentration. This will subsequently give you the largest change in signal for the smallest alteration in the AMP concentration

Figure 4. Phosphodiesterase titration curve.

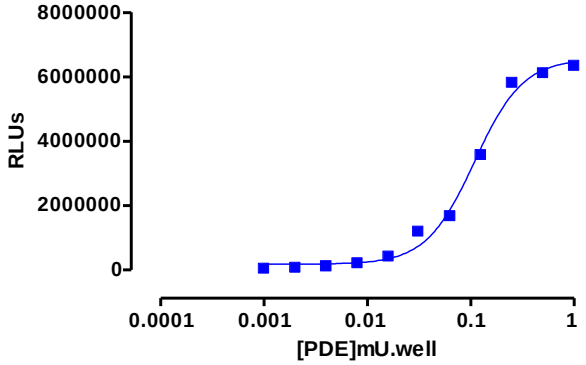


Figure 4. 3‘,5‘ cyclic nucleotide phosphodiesterase (Sigma P0520, isoform unspecified) activity was measured using the method outlined in the protocol. The assay was performed with 10 µM cAMP and incubated for 30mins at RT. An EC_{50} of ~0.1 [PDE]mU per well was determined.

Running a screen

The following is a suggested method for running a screen in either 96 or 384 well formats using Protocol A.

1. Add 10 µl of 40 µM inhibitor to each well in compound solvent i.e. 10% (v/v) DMSO.
2. For control wells use compound solvent only.
3. To all wells add 10 µl of phosphodiesterase diluted in reaction buffer at 4 x optimal concentration.
4. To all wells add 20 µl of cAMP at 2 x optimal concentration.
5. Incubate for 30-60 minutes at room temperature.
6. Add 10 µl of Stop Solution (optional).
7. Add 20 µl of AMP Detection Reagent.
8. Incubate for at least 10 minutes at room temperature.
9. Measure luminescence using a 0.1 second integration time.

Figure 5. Percentage inhibition of 150 pharmacologically active compounds.

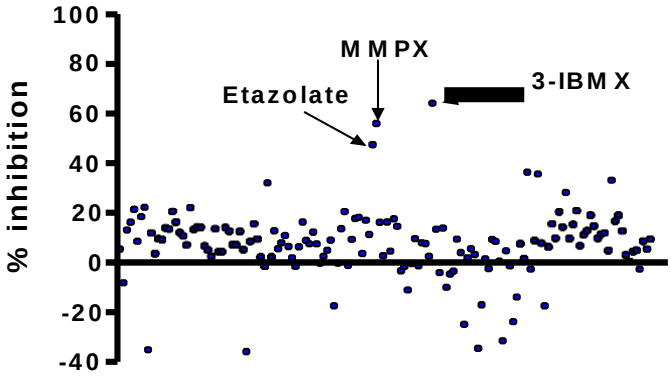


Figure 5. A focused library containing 150 pharmacologically active compounds (10 µM in 2.5% (v/v) DMSO) was screened using the method outlined above on 3‘,5‘ cyclic nucleotide phosphodiesterase (0.1 µU per well). Using a cut off of 40% inhibition the compounds 3-IBMX, MMPX and Etazolate were shown to inhibit this phosphodiesterase.

Table1. Compounds scored as hits.

Compound	% inhibition
3-IBMX	64
MMPX	56
Etazolate	47

Determining IC_{50} values

The following is a suggested method for running IC_{50} analysis using Protocol A, in a 96 well plate format.

1. Produce a twofold dilution series of the inhibitor across the plate, final volume 10 µl.
2. To all wells add 10 µl of the phosphodiesterase diluted in assay buffer at 4 x optimal concentration.
3. For control wells use phsophodiesterase reaction buffer only.
4. To all wells add 20 µl of cAMP at 2 x optimal concentration.
5. Incubate for 30-60 minutes at room temperature.
6. Add 20 µl of AMP detection reagent.
7. Incubate for at least 10 minutes at room temperature.
8. Measure luminescence using a 0.1 second integration time.

Figure 6. IC_{50} analysis of 3-IBMX.

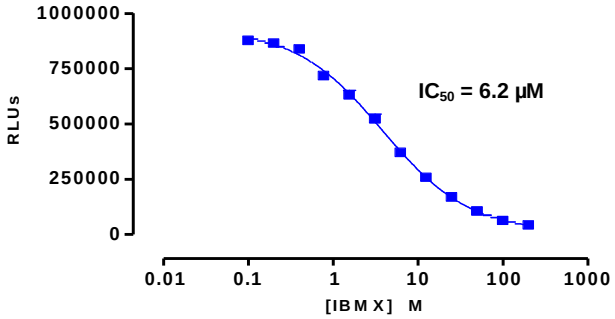


Figure 6. IC_{50} analysis of 3-IBMX (0.1-200 µM), on 3‘,5‘ cyclic nucleotide phosphodiesterase (0.1 µU per well) yielded a value of 6.2 µM.

Z’ analysis

The following is a suggested method for Z’ factor analysis using Protocol A, in a 96 well plate format.

1. To the first six columns of a 96 well plate add the phosphodiesterase at 2 x optimal concentration diluted in reaction buffer in a total volume of 20 µl.
2. Add the same volume of reaction buffer (minus phosphodiesterase) to the remaining 6 columns.
3. To all wells add 20 µl of cAMP at 2 x optimal concentration to initiate the reaction.
4. Incubate for 30-60 minutes at room temperature.
5. Add 20 µl of AMP detection reagent.
6. Incubate for at least 10 minutes at room temperature.
7. Measure luminescence using a 0.1 second integration time.

Figure 7. Z’ Analysis.

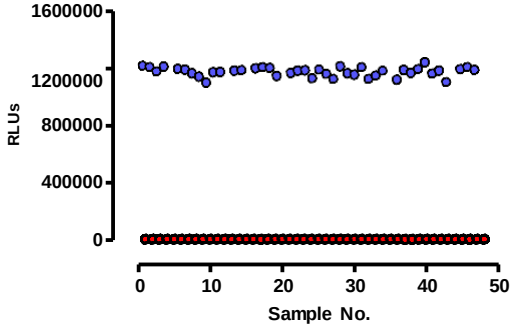


Figure 7. Z’ factor analysis was performed in a 96 well plate using 3‘,5‘ cyclic nucleotide phosphodiesterase (0.1 µU per well) in half the wells, buffer in the remainder. Total reaction volume 40 µl, [cAMP] 10 µM. Z’ factor = 0.86.

Interpretation of results

Due to the convenience of bioluminescent measurement the PDELight™ Kit offers an easily established end point for the detection of cAMP dependant phosphodiesterases. The bioluminescent signal produced by the luciferase reaction is directly proportional to the activity of the phosphodiesterase.

Phosphodiesterase activity will result in the conversion of cAMP to AMP and an increased bioluminescent signal compared with the controls. An inhibiting compound will reduce the conversion of cAMP to AMP and will therefore reduce the light output of the assay in comparison to the inhibitor free controls.

In most cases the direct output from the luminometer (commonly relative light units or RLUs) or beta counter (cpm) can be used to assess the enzymatic response to the target compound. This reaction is linear between 0.5 nM-20 µM of AMP.

Trouble shooting / main considerations

Reaction volume

A 40 l total volume for the phosphodiesterase reaction is recommended. This will enable the use of low volumes of valuable components such as the phosphodiesterase. This volume is recommended for both 96 and 384 well formats. If a 1536 format is required please contact technical support for further advice (see back page for contact details). It is essential that the volume: volume ratio of the AMP detection reagent to the reaction mixture is maintained at 1:2 respectively if alterations to the assay are necessary. If stop solution is to be used a ratio of 1:4, stop solution to reaction mixture volume should be used.

Integration time

A 0.1 second integration time is highly recommended due to the high light output of the assay. If adjustment is needed please keep in the range 0.1 – 1 second integrated.

AMP concentration

The assay is linear up to 20 µM AMP. Therefore, the final AMP concentration should be less than 20 µM .

Interferences

The PDELight™ Assay is designed to be resistant to DMSO up to a final concentration of 10% (v/v) and many other interfering compounds. Intensely red colored compounds will quench light emission and should be taken into consideration. The luciferase reaction requires Mg++ as a co-factor therefore chelating agents such as EDTA should be avoided in high concentrations.

Plate recommendations

White walled, white or clear bottom plates suitable for luminescence need to be used. Black plates can be used if excess light is a problem.

High background levels

Reaction components such as buffer, phosphodiesterase, cAMP, plates and other materials should be free from ATP contamination. To avoid such contamination the use of gloves is recommended when handling the PDELight™ AMP detection reagent.

Luciferase inhibition

In general it has been found that inhibitors of phosphodiesterases are not inhibitors of luciferase. To counter false negatives produced from luciferase inhibition a counter screen may be required. Figure 8, shows 8 known PDE inhibitors and their effects on luciferase.

Figure 8, Effects of known PDE inhibitors on luciferase.

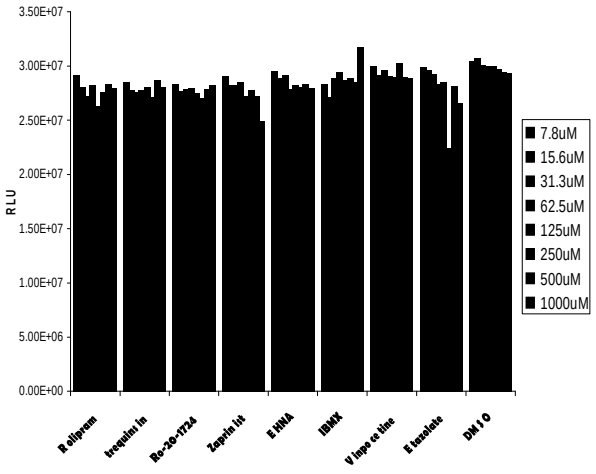


Figure 8. 40 µl of each compound in 10% (v/v) DMSO was diluted in assay buffer to give concentrations ranging from 7.8 µM to 1000 µM. To each well 40 µl of 10 µM ATP was added and 40 µl of PDELight™ AMP detection reagent, LT27-250. Measurements were made after 30 minutes. There was no significant change in signal after 30 minutes exposure of the luciferase to the increasing concentrations of the inhibitors.

References

Conti M, Richter W, Mehats C, Livera G, Park JY, Jin C: (2003) Cyclic AMP-specific ODE4 phosphodiesterases as critical components of cyclic AMP signalling. *J Biol Chem* **Vol:**278(8) p5493-6.

Conti M:(2000) Phosphodiesterases and cyclic nucleotide signalling in endocrine cells. *Mol Endicrinol* **Vol:**14(9) p 1317-27.