Cyclic Nucleotide Phosphodiesterase Assay Kit
A BIOMOL GREEN™ Quantizyme™ Assay System

Instruction Manual
BML-AK800

For research use only
BACKGROUND

The BIOMOL Cyclic Nucleotide Phosphodiesterase (PDE) Assay Kit 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 BIOMOL GREEN™ 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 IC₅₀ of approximately 25 µM.

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 the instructions for standard curve preparation on page 2.

The assay offers the following advantages:

NON-RADIOACTIVE
CONVENIENT ONE STEP DETECTION
MICROPLATE FORMAT

Individual components of this kit are available separately as below:

<table>
<thead>
<tr>
<th>Component</th>
<th>ELS cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIOMOL GREEN Reagent</td>
<td>BML-AK111</td>
</tr>
<tr>
<td>IBMX</td>
<td>BML-PD140</td>
</tr>
<tr>
<td>Phosphodiesterase (from bovine brain)</td>
<td>BML-KI456</td>
</tr>
<tr>
<td>3',5'-cAMP Substrate</td>
<td>BML-KI180</td>
</tr>
<tr>
<td>5'-AMP Standard</td>
<td>BML-KI184</td>
</tr>
<tr>
<td>5'-GMP Standard</td>
<td>BML-KI182</td>
</tr>
<tr>
<td>Desalting column and resin</td>
<td>BML-KI100</td>
</tr>
<tr>
<td>1/2-Volume Microplate</td>
<td>BML-KI101</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>BML-KI307</td>
</tr>
</tbody>
</table>


PLEASE READ ENTIRE BOOKLET BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT BIOMOL TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

COMPONENTS

NOTES ON STORAGE: For highest stability, store all components, except microplate (KI-101), at -70°C. Because its stability when frozen in solution is poor, the PDE enzyme (KI-456) 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 Preparing assay reagents, p. 2). The 5'-nucleotidase (KI-307) is used undiluted at 10 µl 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.

BML-KI456-0020 PDE ENZYME (from bovine brain)
FORM: Lyophilized solid, 4 U per vial.
1 U = 1 nmol 3',5'-cAMP to 5'-AMP per minute under the conditions of the linearity assay on p. 3 (30°C, pH 7.4, 200 µM 3',5'-cAMP (KI-180)).
STORAGE: -70°C; AVOID FREEZE/THAW CYCLES!
QUANTITY: 5 x 4 U
PRESENTATION: screw-cap microfuge tube

BML-KI307-5000 5'-Nucleotidase (from Crotalus atrox venom)
FORM: 5 kU/µl in 10 mM Tris-HCl, pH 7.4, 125 mM NaCl, 1 mM MgCl₂, 10% (v/v) glycerol
One U will release 1 pmol phosphate per minute from 200 µM 5'-AMP, 30°C in a reaction buffer of 10 mM Tris-HCl, pH 7.4, 0.2 mM MgCl₂
STORAGE:-70°C
QUANTITY: 1 ml
PRESENTATION: screw-cap microfuge tube

BML-KI180-0002 3',5'-cAMP SUBSTRATE
FORM: 1 mM in assay buffer (10 mM Tris-HCl, pH 7.4)
STORAGE: -20 or -70°C
QUANTITY: 2 ml
PRESENTATION: screw-cap microfuge tube

BML-KI184-0001 5'-AMP STANDARD
FORM: 100 µM in 10 mM Tris-HCl, pH 7.4
STORAGE: -70°C
QUANTITY: 40 ml
PRESENTATION: liquid in screw-cap plastic bottle

BML-AK111-9090 BIOMOL GREEN™ REAGENT
STORAGE: 4°C. Long-term at -70°C
QUANTITY: 20 ml
PRESENTATION: liquid in screw-cap plastic bottle

BML-KI184-0001 5'-AMP STANDARD
FORM: 100 µM in 10 mM Tris-HCl, pH 7.4 buffer
STORAGE: -20 or -70°C
QUANTITY: 1.0 ml
PRESENTATION: screw-cap microfuge tube
BML-KI182-0001  5'-GMP STANDARD  
FORM: 100 µM in 10 mM Tris-HCl, pH 7.4 buffer  
STORAGE: -20 or -70°C  
QUANTITY: 1.0 ml  
PRESENTATION: screw-cap microfuge tube  

BML-PD140-9090 INHIBITOR (IBMX)  
FORM: 200 µM in assay buffer (10 mM Tris-HCl, pH 7.4)  
STORAGE: -20 or -70°C  
QUANTITY: 200 µl  
PRESENTATION: screw-cap microfuge tube  

BML-KI100-0001 DESALTING COLUMN AND RESIN  
FORM: 5 ml polypropylene disposable column and P6 DG desalting resin  
STORAGE: RT. After rehydration store at 4 °C.  
QUANTITY: 1 column and 1 g of resin  

80-2404 1/2 VOLUME MICROPLATE  
STORAGE: Room temperature.

OTHER MATERIALS REQUIRED  

Microplate reader capable of measuring A$_{620}$ to ±3-decimal accuracy.  

Pipetman capable of pipetting 5-100 µl accurately  

Multi-channel pipetman capable of pipetting 100 µl (optional).  

Ice bucket to keep reagents cold until use.

EXPERIMENTAL METHODS  

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 BEING INVESTIGATED, AND MUST BE DETERMINED BY THE INDIVIDUAL USER. NO WARRANTY OR GUARANTEE OF PERFORMANCE USING THESE PROCEDURES IS MADE OR IMPLIED.

PRECAUTIONS: The BIOMOL GREEN™ 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 dH$_2$O 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) as present in the BIOMOL GREEN™ reagent) in the high speed supernatant (HSS) extract.  

1. Rehydrate Desalting Column Resin (BML-KI100) in a 50 ml conical tube by adding 20 ml of phosphate free dH$_2$O and vortexing briefly. Allow to set for 4 hours at RT or overnight at 4 °C.  
2. Decant the dH$_2$O carefully, then add fresh dH$_2$O at a 1:1 ratio to the rehydrated resin (BML-KI100) (~10 ml).  
3. Add rehydrated resin (KI-100) to the Chromatography Column (BML-KI100) to obtain a 5 ml settled-bed volume (~5.5 cm bed height). Remove tip from column and allow dH$_2$O to drain by gravity.  
4. Equilibrate column by adding 8 ml of assay buffer (BML-KI181) 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 -70 °C.

TIP: The effective removal of phosphate/nucleotides from the extract should be tested qualitatively by adding 100 µl BIOMOL GREEN™ reagent to 1 µl extract, and a separate sample of 1 µl dH$_2$O. 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 inhibitor, 5’-AMP and/or 5’-GMP standard. Store all on ice.  
2. Prepare 20 U/ml solution of PDE (BML-KI456) 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 BIOMOL GREEN™ Reagent to room temperature.

Preparing a standard curve:  

1. Prepare two dilutions in PDE Assay buffer (BML-KI181), 75 µM and 50 µM, using either the 5’-AMP (BML-KI184) or 5’-GMP (BML-KI182). For example, bring aliquots of 150 µl and 100 µl 5’-AMP Standard (BML-KI182) and 200 µl with assay buffer.  
2. Using PDE Assay Buffer, prepare two sets of 1:1 serial dilutions of the 75 µM and 50 µM 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 µ 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 thoroughly and then remove 40 µl and discard.  
   e) Remove 40 µl from well C and add it to well E. Mix well E thoroughly and then remove 40 µl and discard.  
   f) Add 40 µl 1X assay buffer to wells C through H.  
   g) Remove 40 µl from well B and add it to well D. Mix thoroughly by pipetting up and down several times.  
   h) 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’.**  
   i) Add 40 µl of 5’-nucleotidase (BML-KI307, undiluted, 5 kU/µl) to each well and mix thoroughly.  
   j) Incubate at 30°C for 30 minutes.  
   k) Proceed to section titled “To terminate reactions:” on page 3 of kit booklet.
To prepare a time course/linearity assay:
1. Dilute cAMP substrate (BML-KI180) to 0.5 mM with assay buffer (BML-KI181).
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 (BML-KI181) to each well.
4. Add 10 µl of 5'-nucleotidase (KI-307, 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 at 20 U/ml. (See “Preparing assay reagents”, p. 2.) 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 (BML-KI456). 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.

<table>
<thead>
<tr>
<th>Sample Well</th>
<th>5'-AMP/5'-GMP Standard Curve</th>
<th>Time course Min. (Columns 3,4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.0</td>
<td>90</td>
</tr>
<tr>
<td>B</td>
<td>2.0</td>
<td>60</td>
</tr>
<tr>
<td>C</td>
<td>1.5</td>
<td>45</td>
</tr>
<tr>
<td>D</td>
<td>1.0</td>
<td>30</td>
</tr>
<tr>
<td>E</td>
<td>0.75</td>
<td>20</td>
</tr>
<tr>
<td>F</td>
<td>0.50</td>
<td>10</td>
</tr>
<tr>
<td>G</td>
<td>0.25</td>
<td>5</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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

To terminate reactions:
1. After incubating wells for desired duration, terminate reactions by addition of 100 µl BIOMOL GREEN™ Reagent (# BML-AK111-9090). 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.
4. Perform data analysis (see below).

NOTE: Retain microtiter plate for future use of unused wells!

TABLE 1. EXAMPLE OF STANDARD CURVE AND TIME COURSE/LINEARITY MICROPLATE SAMPLES.

<table>
<thead>
<tr>
<th>Substrate (0.5 mM)</th>
<th>Assay Buffer</th>
<th>5'-Nase. (5 kU/µl)</th>
<th>Test cpd</th>
<th>PDE (4 mU/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>20 µl</td>
<td>15 µl</td>
<td>10 µl</td>
<td>0 µl</td>
</tr>
<tr>
<td>TEST</td>
<td>20 µl</td>
<td>5 µl</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>IBMX</td>
<td>20 µl</td>
<td>5 µl</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

To prepare a test sample/inhibitor assay:
1. Prepare samples containing PDE, substrate and test compound dissolved in assay buffer (BML-KI181) as listed in Table 2. Include the IBMX inhibitor if desired.
2. Incubate samples at appropriate temperature (e.g.: 37°C) and time (e.g.: 60 min).

<table>
<thead>
<tr>
<th>Substrate (0.5 mM)</th>
<th>Assay Buffer</th>
<th>5'-Nase. (5 kU/µl)</th>
<th>Test cpd</th>
<th>PDE (4 mU/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>20 µl</td>
<td>15 µl</td>
<td>10 µl</td>
<td>0 µl</td>
</tr>
<tr>
<td>TEST</td>
<td>20 µl</td>
<td>5 µl</td>
<td>10 µl</td>
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<td>IBMX</td>
<td>20 µl</td>
<td>5 µl</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

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.

DATA ANALYSIS

5'-AMP or 5'-GMP Standard Curve
1. Plot standard curve data as OD_{620nm} versus nmol 5'-AMP or 5'-GMP (see Figure 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'-AMP released = (OD_{620nm} – y-intercept)/slope
(See SAMPLE CALCULATION below.)
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.

SAMPLE CALCULATION:
Best-fit eqn.: OD_{620nm} = 0.232(nmol 5'-AMP) + 0.0709
Rearranged eqn.: nmol 5'-AMP = (OD_{620nm} - 0.0709)/0.232
Example: An unknown produces an OD_{620nm} = 0.400
5'-AMP released = (0.400 - 0.0709)/0.232 = 1.42 nmol

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.

Figure 1. Standard Curve for 5'AMP. Duplicate wells of 5'-AMP dilutions were prepared as described (see Preparing a standard curve, p.2). 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 BIOMOL Green (100 µl/well). After 30 min., the phosphate-dependent color reaction was measured by reading OD_{620nm} in a microplate-reading spectrophotometer.
**Application Examples**

**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_{620} > 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 BIOMOL Green 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.
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