DRG® Cyclic GMP (EIA-2097)

Revised 15 Feb. 2011 rm (Vers. 2.1) **RUO**

This kit is intended for Research Use Only.

Not for use in diagnostic procedures.

Description

The DRG Cyclic GMP (cGMP) EIA system provides a simple, sensitive method for measuring cGMP in biological samples at a range of 0.05 to 100 picomoles/ml. The method is based on the competitive binding by cGMP and an alkaline phosphate derivative of cAMP for a limited amount of specific antibody. The amount of enzyme-labelled cGMP bound to antibody decreases with increasing concentration of cGMP. Separation of bound cGMP from free cGMP is achieved by a second antibody bound to the sides of 96-well (8 well strips) plates.

There are two plates and sufficient reagents for 192 standards and samples.

Summary of Test

The DRG Cyclic GMP EIA has been designed for the 96-well format. Specific antibody and the enzyme-cAMP conjugate are combined with standards or unknowns in the plates and incubated 18-24 hrs. at 4ºC or 3 ½ hours at room temperature. After washing the plate, enzyme activity is determined and unknowns calculated from a standard curve prepared at the same time.

References

1. Sutherland, E.W. and Rall, T.W., J.A.C.S. 79, 3808 (1957).
2. Cook, W.H., Lipkin, D. and Marham, R. ibid 79, 3607.
3. Sutherland, E.W., Phosphorous Metabolism, edited by W.E. McElroy and B. Glass, Vol. Johns Hopkins Press (1951).
4. Goldberg, M.L. Clin. Chem. 23, 576 (1977).
5. Watling, K.J. and Dowling, J.E., J. of Neurochemistry 41, 1205 (1983).
6. Advances in Cyclic Nucleotide Research Vol. I, Greengard, P. and Robism, R.A., Raven Press, New York.
7. ibid.
8. Steiner, A.L. Methods in Enzymology 38, Hardman, J.G. and O'Malley, B.W., Academic Press, New York (1974).
9. Farmer, R.W., Harrington, C.A. and Brown, G.H., Anal Biochem. 64, 455 (1975).
10. Broadus, et al, Annal. N.Y. Acad. Sci. 185, 50 (1971).
11. Harper, J.F. and Brooker, G., J. Cyclic Nucleotide Res. 1, 207-218 (1975)

FOR RESEARCH USE ONLY

Reagents: Description and Preparation

Store all reagents at 4°C up to 6 months except as noted.

1. Sodium Acetate Concentrate. One vial. Dilute contents to 500ml with distilled or deionized water (stable at least 2 months at 4°C).
2. Cyclic GMP-Alkaline Phasphatase Conjugate. Two vials. **Store at -20°C up to 6 months or at 4°C for 1 month.** For one plate reconstitute one vial with acetate buffer. For the whole kit reconstitute both vials and combine them. Reconstitute with volume given on the label. The solution is stable for 2 months at -20°C.
3. Cyclic GMP Standard. One vial**.** Contains 5000 picomoles, lyophilized. Add exactly 5.0ml acetate buffer. Stopper, mix thoroughly. Stable for 2 months at 4°C.
4. Cyclic GMP Antiserum. One vial. Contains 12ml specific cAMP antiserum. Stable 6 months at 4°C.

5. Acetic Anhydride. One vial, 1ml neat liquid, moisture sensitive. **Caution:** corrosive liquid, lacrymator, combustible. Do not inhale or ingest. Use in a fume hood.
6. Triethylamine. One vial. Contains 2ml. Must be kept dry. **Caution:** flammable, toxic. Do not breathe vapors.
7. p-Nitrophenyl Phosphate. (PNPP). One vial. Store dry at 4°C.
8. Diethanolamine Buffer. One vial. Store at 4°C. Use with PNPP for determination of enzyme activity.
9. 5x Wash Buffer Concentrate. One vial. Contains 100ml. Dilute contents to 500ml with deionized water.
10. Two 96-Well strip plates. Plates are coated with second antibody. Store dry at 4°C.

Preparation of Samples

Several techniques are available for the extraction and/or partial purification from various tissues [6], fluids such as plasma[4], cell extracts[5], and tissue culture medium[7]. Since endogenous phosphodiesterases are likely contaminants of most biological samples, a denaturing, deproteinizing treatment is commonly employed. These include treatment with trichloroacetic acid[8], ethanol[9], perchloric acid [10], followed by some chromatography such as ion-exchange or alumina. Samples should not contain metal chelators, e.g., EDTA or EGTA.

A. Tissue Culture Extracts or Media (Acetylate for all formats)

1. Intracellular cAMP

a) Monolayers. Wash the plates 3 times with physiological saline (Ca^{++},Mg^{++} free). Obtain a cell count. Flood the cells with 5% trichloroacetic acid. After 5 minutes, remove supernatant, wash once with water. Combine TCA supernatant with water wash and extract with ether as in C. Lyophilize if necessary.

b) Suspensions. Wash the cells with PBS by centrifugation in polypropylene tubes, add 0.05M HCl, and place the tubes in boiling water 3 minutes. Cool and lyophilize. Reconstitute with acetate buffer, filter or centrifuge to clarify.

2. Extracellular cAMP. Tissue culture media needs to be diluted at least 5 fold with acetate buffer. Store at -20ºC or lower.

B. Urine

Store at –20ºC or lower. Dilute urine 100 fold with acetate buffer just prior to assay. Use100ul/tube. Use non-acetylated protocol.

C. Tissue Extraction[11]

Homogenize 1 part (by weight) with 9 parts 5-10% trichloroacetic acid with an instrument such as a Polytron (Brinkman Instruments). Clarify by centrifugation and extract the supernatant with five volumes of water-saturated ether in a screw cap centrifuge tube (centrifugation may be used to separate the layers if necessary). Remove the ether layer and repeat extraction of aqueous layer two times. Remove residual ether from the aqueous layer. The sample may be heated up to approximately 50°C.

D. Plasma Acetylation[11]

Heparinized or citrated plasma is brought to 0.5mM IBMX, cooled in an ice bath and assayed immediately or stored at –70°C.

Procedure:
Add 950ul acetate buffer to 50ul of specimen.
Use 210ul per tube. Continue with acetylation protocol.

DRG® Cyclic GMP (EIA-2097)

Revised 15 Feb. 2011 rm (Vers. 2.1) **RUO**

Protocol for Non-Acetylated Samples
Working cAMP Standards should be made up from the stock solution just prior to the assay. Prepare new standards each day.

Sample protocol for working standards. (Vortex after each addition)		pmole/ml
A. 0.1ml stock standard	+ 0.9ml working buffer	100
B. .05ml stock standard	+ 0.95ml working buffer	50
C. 0.2ml solution A	+ 0.8ml working buffer	20
D. 0.1ml solution B	+ 0.9ml working buffer	5
E. 0.1ml solution C	+ 0.9ml working buffer	2
F. 0.1ml solution D	+ 0.9ml working buffer	0.5

Procedure: Allow solutions to warm to room temperature prior to setting up the assay.

1. Remove strips not used from the frame, store dry at 4ºC. Use 16 wells for blank, maximum binding, (Bmax), and 6 standards in duplicate, and at least 2 wells for each unknown.

2. Add 150ul buffer for the blanks, 100ul buffer for Bmax and 100ul per well of each standard and unknown to appropriate wells.

3. Add 50ul of diluted CAMP-Alkaline Phosphatase tracer to all wells and 50ul cAMP antiserum to all wells, except the blanks. Incubate 3 ½ hours at room temperature or 18-24 hours at 4ºC.

4. Aspirate all wells and fill with diluted Wash Buffer, aspirate completely. Repeat for a total of 5 washes (See Note 1).

5. Tap the wells gently on paper towels to remove remaining liquid. Add 200ul PNPP, 4mg/ml in Diethanolamine Buffer to all wells (cover tightly with new sealing tape). Incubate approx. 1 hr. at 37ºC. Remove sealing tape, measure absorbance at 405-410nm. However, data may be taken at any time. (Do not allow absorbance of the Bmax to exceed 1.5). Extend incubation, if necessary.

Calculations:

1. Determine mean values for each set of points.

2. Obtain mean net absorbance by subtracting the averaged nonspecific binding from the means of all standards and unknowns.

3. Calculate relative bindings (%B/Bo). Divide net mean absorbance of each standard and unknown by net mean absorbance of the zero standard multiply by 100.

4. Construct a standard curve by plotting %B/Bo (from step 3) versus pmole cAMP/ml. Four cycle semi-logarithmic paper (smooth curve) or four cyclic logarithmic-logistic paper (straight line) may be used.

5. Determine pmoles/ml for each unknown.

Revised 15 Feb. 2011 rm (Vers. 2.1) **RUO**

Protocol for Acetylated Samples

Prepare working standards in the range of 0.01 to 10pmoles/ml. Start by making a 100pmoles/ml solution. Prepare 6 standards as shown below. Additional standards may be added if desired.

		Pmole/ml
A. 100ul of 100pmoles/ml solution	+ 0.9ml working buffer	10
B. 100ul of 100pmoles/ml solution	+ 1.9ml working buffer	5
C. 250ul solution A	+ 0.75ml working buffer	2.5
D. 100ul solution B	+ 0.9ml working buffer	0.5
E. 100ul solution C	+ 0.9ml working buffer	0.25
F. 100ul solution D	+ 0.9ml working buffer	0.05

Prepare fresh solutions each day.

Procedure:

1. Prepare Bmax, standards (above) and unknowns as previously described. Transfer 420ul acetate buffer (blank and Bmax) and 210ul of each standard and unknown to labeled 12x75mm glass tubes.

2. Prepare a fresh solution of 1 part acetic anhydride, 2 parts triethylamine immediately preceding addition. Add 10ul of this solution to all tubes and 20ul to the blank and Bmax tube. The acetylating reagent must be added directly into test solution and immediately vortex mixed. **Note:** The reagent is very unstable. Mix only enough to last 3-5 minutes (approximately 50-60 tubes).

3. Let stand 30 mins. Transfer 100ul (in duplicate) of each solution to a well, including blank and Bmax. Add 50ul acetate buffer to blank wells.

4. Proceed with Non-Acetylated Protocol starting at Step 3.

   Incubation with substrate should be 2 hours at 37ºC.

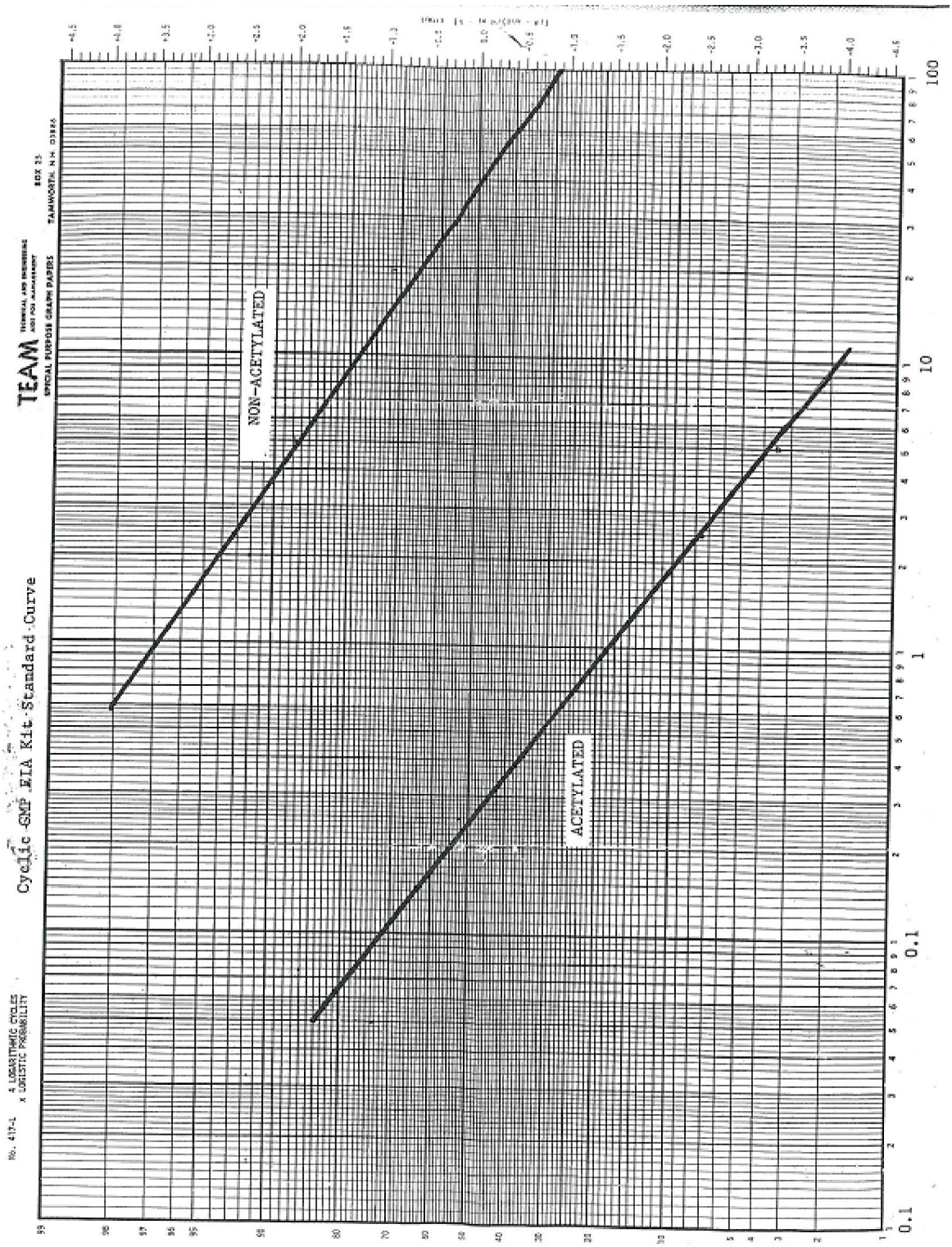
***NOTE 1**

Samples that could contain large quantities of endogenous alkaline phosphatase should be evaluated for possible interference. It may be necessary to do additional washing and/or deproteinize the sample. A "sample blank" (i.e., no specific antibody) should be done on such samples.

Sample Data

Non-Acetylated	Sample ID	Absorbance 410mm		Net Mean Absorbance	%B/B$_0$
	Blank	0.124	0.113	0.118	
Standard	0 pmole/ml (B$_0$)	0.823	0.774	0.680	100
Standard	0.5 pmole/ml	0.806	0.764	0.667	98
Standard	2pmole/ml	0.754	0.740	0.629	92
Standard	5pmole/ml	0.751	0.681	0.598	88
Standard	20pmole/ml	0.624	0.613	0.500	73
Standard	50pmole/ml	0.553	0.506	0.411	61
Standard	100pmole	0.380	0.355	0.250	37

Acetylated	Sample ID	Absorbance 410mm		Net Mean Absorbance	%B/B$_0$
	Blank	0.117	0.119	0.118	
Standard	0 pmole/ml (B$_0$)	0.606	0.675	0.552	100
Standard	0.05pmole/ml	0.528.	0.527	0.410	74
Standard	0.25pmole/ml	0.409	0.408	0.296	52.7
Standard	0.5pmole/ml	0.283	0.287	0.167	30
Standard	2.5pmole/ml	0.161	0.159	.042	7.6
Standard	5pmole/ml	0.139	0.136	.020	3.5
Standard	10pmole/ml	0.125	0.130	.010	1.8



Version 02/09~rm