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INSTRUCTION MANUAL CYCLIC GMP RIA KIT

Catalog No. IB78173

Introduction

Cyclic GMP (cGMP), like Cyclic AMP, mediates numerous cellular processes and is ubiquitous in nature. Also analogous to cAMP, a specific enzyme, guanyl cyclase is activated in response to a primary or initial response, such as the binding of a hormone to the target cell. This results in an increase in intracellular cGMP. However, in many instances, increased cGMP concentrations leads to responses which are antagonistic to those of cAMP. The cyclic nucleotides have often been referred to as secondary messengers^{1,2,3}.

Description

This cyclic GMP (cGMP) radioimmunoassay system provides a simple, sensitive method for measuring cGMP in biological samples at a range of 0.05 to 1000 pmol/ml, (0.005-100 pmol/tube).

The method is based on the competitive binding of cGMP in the sample and a radioiodinated derivative of cGMP ([I-125] cGMP), for a highly specific antibody. The amount of labeled cGMP found in the complex decreases with increasing concentration of unlabeled cGMP in the sample. Separation of antibody bound cGMP from free cGMP is achieved through a precipitating antibody incorporated in the reagent system. Determination of the unknown is made by the comparison with a standard curve constructed in the same fashion.

Sufficient reagents are included to do 200 tubes including standards.

Summary of Test

This cyclic GMP Radioimmunoassay Kit employs a pre-conjugated double antibody separation system in an acetate buffer. Components of the system have been combined to minimize pipeting steps (3) and incubation (1). Standards and unknowns are combined with tracer solution and antibody. Solutions are incubated 18-20 hours at 4°C. One ml acetate buffer is added, the tubes centrifuged and the visible pellets separated from supernatant. Radioactivity in the precipitate is counted and unknowns are determined from a standard curve.

FOR RESEARCH USE ONLY (REV. 09/05)

References:

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Precautions

Radioactive Materials - Use, Handling, Storage and Disposal of Wastes.

This radioactive material may be received, possessed and used only by research laboratories for in vitro laboratory tests not involving external or internal administration to humans or animals. Its receipt, acquisition, possession, use and transfer are subject to regulation and a general license of the U.S. Nuclear Regulatory Commission or of a state with which the Commission has entered into an agreement for the exercise of a regulatory authority.

Safety Precautions

1. Do not drink or smoke in areas where radioactive materials are used.
2. Do not pipet radioactive solutions by mouth.
3. Wash hands following use of radioactive materials.
4. Radioactive materials should be used only in designated areas.
5. All radioactive materials should be disposed of according to regulations of the agencies with jurisdiction over the laboratory.
6. Spills should be handled in accordance with established procedures.
7. Store radioactive compounds in clearly marked containers in predesignated areas.

Preparation of Samples

Several techniques are available for the extraction and/or partial purification from various tissues⁶, fluids such as plasma⁴, cell extracts⁵, and tissue culture medium⁷. Since endogenous phosphodiesterases are likely contaminants of most biological samples, a denaturing, deproteinizing treatment is commonly employed. These include treatment with trichloroacetic acid⁸, ethanol⁹, perchloric acid¹⁰, followed by some chromatography such as ion-exchange or alumina.

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

1. Intracellular cGMP

- 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. The combined supernatants are extracted 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 cGMP. Tissue culture media needs to be diluted at least 5 fold with acetate buffer.

B. Urine

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

C. Tissue Extraction¹¹

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 .

$[\text{H}^3]$ cGMP, **Catalog No. DEBT349** should be used to determine recoveries. Be sure to account for the added mass of cGMP if this is done.

D. Plasma Acetylation¹¹

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

Procedure:

1. Add 950ul acetate buffer to 50ul of specimen.
2. Use 100ul per tube. Continue with acetylation protocol.

Reagents: Description & Preparation

1. Tracer Diluent. **Catalog No. DEBT332.** One vial. Contains sodium acetate buffer (pH 6.2), normal rabbit IgG, phosphodiesterase inhibitors and sodium azide. Store at 4°C. Stable 2 months.
2. cGMP Tracer Concentrate. **Catalog No. DEBT341R.** Two vials. Each contains approximately 1.5uCi (at calibration date) [125 I] succinyl cGMP-tyrosine methyl ester (sc-cGMP-TME-I- 125 I) diluted in 50% n-propanal-water. Add 10.5ml of Tracer Diluent, stopper, mix by inversion. Store at 4°C. Stable 2 months from production date.
3. Sodium Acetate Concentrate. **Catalog No. DEBT333.** One vial. Dilute contents of vial to 500ml with distilled or deionized water for working buffer. Final solution 0.05M sodium acetate (pH 6.2). Store at 4°C. Stable for at least 4 weeks.
4. Cyclic GMP Standard. **Catalog No. DEBT344.** One vial. Contains 5000 picomoles, lyophilized. Add 5ml of working buffer with a volumetric pipet, stopper and mix thoroughly. Stable 4 weeks at 4°C.
5. Cyclic GMP Antiserum. **Catalog No. DEBT342P.** Two vials containing specific cGMP antiserum for 100 tubes. Also contains goat anti-rabbit IgG. Store at 4°C. Stable 6 months.

Note: Cyclic GMP Antiserum is in the form of a fine suspension, which may settle on prolonged standing. Always mix the solution, by inversion several times, prior to addition of the antiserum. Mixing is recommended after every 50 additions.

6. Nonspecific Binding Reagent. **Catalog No. DEBT335.** One vial. Contains 1ml, sufficient for 5 standard curves. Prepared as cGMP antiserum, but without specific antiserum. Stable 2 months at 4°C.
7. Acetic Anhydride. **Catalog No. DEBT336.** One vial. Contains 1ml. Moisture sensitive. Do not open cool vials or use wet pipets. **Caution:** Corrosive liquid - Lacrymator Combustible Liquid.
8. Triethylamine. **Catalog No. DEBT337.** One vial. Contains 2ml. Must be kept dry. Do not open cool vials. **Caution:** Flammable. Toxic, do not breath vapors.

Specificity: Expressed as (pmol cGMP at 50% Bmax/pmol test ligand at 50% Bmax).

c-GMP	1
c-AMP	715
GMP	$>10^6$
ATP	$>10^7$
GDP	$>10^7$
GTP	$>10^7$

Protocol for Non-Acetylated Samples

Working 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).

	<u>pmol/ml</u>	<u>pmol/tube</u>
A. 0.2 ml stock standard + 0.8 ml working buffer	200	20
B. 0.1 ml stock standard + 0.9 ml working buffer	100	10
C. 0.1 ml solution A + 0.9 ml working buffer	20	2
D. 0.1 ml solution B + 1.9 ml working buffer	5	0.5
E. 0.1 ml solution C + 0.9 ml working buffer	2	0.2
F. 0.1 ml solution C + 1.9 ml working buffer	1	0.1

Procedure: Allow solutions, except for acetate buffer, to warm to room temperature prior to setting up the assay.

1. Number 18 12x75 mm glass borosilicate tubes for the standard curve plus at least 2 tubes for each unknown or control.
2. Add 100 μ l working buffer to tubes 3-6. These are the nonspecific binding (3,4) and zero standard or Bo (5,6) tubes. Tubes 1,2 are for total counts.
3. Add 100 μ l working standards, starting with the lowest concentration, in duplicate, to tubes 7-18, as indicated in the summary on page 6.
4. Add 100 μ l unknown samples or controls in duplicate to appropriate numbered tubes. If a volume of sample less than 100 μ l is used, add working buffer to make up the difference. See sample preparation.
5. Add 100 μ l working tracer solution to all tubes.
6. Mix the working antibody solution by gentle inversion 2 or 3 times. (**Note:** Always mix the antibody immediately before pipeting). Pipet 100ul to tubes 5-18 and all additional sample tubes.
7. Mix NSB reagent by gentle inversion and add 100 μ l to tubes 3 and 4.
8. Mix by gently shaking the test tube rack 30-60 seconds. **DO NOT VORTEX** Cover and incubate at 4°C, 18-20 hours.
9. Put aside tubes 1,2 for counting. Add 1 ml of working buffer to the remaining tubes. Vortex. Centrifuge at 2000 x g for 20 minutes at 4°C. (Fixed angle rotors are not recommended).
10. Separate supernatants from pellets by aspiration or decantation. If the latter technique is used, after decanting the supernatant to a waste container, place the inverted tubes in a test tube rack with a paper towel 1-2 minutes. Blot remaining liquid from the rim and set upright.
11. Count all tubes for at least one minute in a gamma spectrometer.

Summary of Non-Acetylated Protocol

<u>Tube No.</u>	<u>Description</u>	<u>Tracer</u>	<u>Buffer</u>	<u>Antibody</u>	<u>NSB Reagent</u>	<u>Unknowns & Standards</u>
1,2	Total Counts	100 µl				
3,4	NSB tubes	100 µl	100 µl		100ul	
5,6	Bo-zero	100 µl	100 µl	100 µl		
7,8	0.1 pmol	100 µl		100 µl		100 µl
9,10	0.2 pmol	100 µl		100 µl		100 µl
11,12	0.5 pmol	100 µl		100 µl		100 µl
13,14	2 pmol	100 µl		100 µl		100 µl
15,16	10 pmol	100 µl		100 µl		100 µl
17,18	20 pmol	100 µl		100 µl		100 µl
19,20	Unknowns	100 µl		100 µl		100 µl
etc.						

Protocol for Acetylated Samples

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

A. 100 µl of 100pmole/ml solution	+ 0.9 ml working buffer	10 pmol/ml
B. 100 µl of 100pmole/ml solution	+ 1.9 ml working buffer	5 pmol/ml
C. 200 µl solution A	+ 0.8 ml working buffer	2 pmol/ml
D. 100 µl solution B	+ 0.9 ml working buffer	0.5 pmol/ml
E. 100 µl solution C	+ 1.9 ml working buffer	0.1 pmol/ml
F. 100 µl solution D	+ 0.9 ml working buffer	0.05 pmol/ml

Prepare fresh solutions each day.

Procedure:

1. Set up total counts, nonspecific binding tubes, zero standard, standards and unknowns as described for steps 1-4 on Non-Acetylated section, using the above standards.
2. Prepare a fresh solution of 1 part acetic anhydride, 2 parts triethylamine immediately preceding addition. Add 5ul of this solution to all tubes except 1 and 2. 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. Proceed with steps of the Non-Acetylated section, starting at Step Number 5.

Calculations:

1. Determine mean values for each set of points.
2. Obtain mean net counts by subtracting the averaged non-specific binding (tubes 3,4) from the means of all standards and unknowns.
3. Calculate relative % binding (%Bo). Divide net mean counts of each standard and unknown by net mean counts of the zero standard (tubes 5 and 6), multiply by 100.
4. Construct a standard curve by plotting % Bo (from step 3) vs pmol cGMP/tube. Four cycle semi-logarithmic paper (smooth curve) or four cycle logarithmic-logistic paper (straight line) may be used.
5. Determine pmol/tube for each unknown. Multiply values by 10 and by dilution factor if appropriate to obtain pmol cGMP/ml.

Example Data

UNACETYLATED

ACETYLATED

<u>Tube No.</u>	<u>Descrip.</u>	<u>cpm</u>	<u>Avg. Net cpm</u>	<u>B/Bo x100</u>	<u>Tube No.</u>	<u>Descrip.</u>	<u>cpm</u>	<u>Avg. Net cpm</u>	<u>B/Bo x100</u>
1	TC	25447	25480		1	TC	25512	25480	
2	TC	25512			2	TC	25447		
3	NSB	288	306		3	NSB	300	319	
4	NSB	324			4	NSB	338		
5	Bo	11989	11944	100	5	Bo	9681	9224	100
6	Bo	12511			6	Bo	9403		
7	0.1 pmol	11000	11108	93	7	.005 pmol	8695	8486	92
8	0.1 "	11827			8	.005 "	8915		
9	0.2 "	10869	10767	90	9	.01 "	7635	7836	85
10	0.2 "	10444			10	.01 "	8675		
11	0.5 "	10444	9901	82.9	11	.05 "	5929	5678	61.5
12	0.5 "	9971			12	.05 "	6065		
13	2 "	8348	8119	68	13	.2 "	3295	3021	32.7
14	2 "	8503			14	.2 "	3386		
15	10 "	5366	5329	44.6	15	0.5 "	1913	1649	17.9
16	10 "	5904			16	0.5 "	2023		
17	20 "	4488	4343	36.4	17	1 "	1291	1004	10.9
18	20 "	4811			18	1 "	1354		



DO NOT USE ABOVE FOR ACTUAL DETERMINATIONS