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HTRF® package insert

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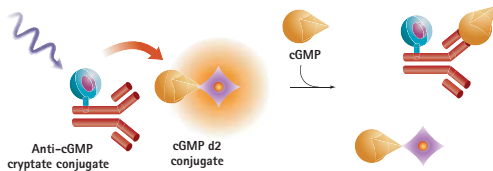
For in vitro research use only
 Storage temperature : 2-8°C

Packaging details :

62GM2PEB	384-well low volume plate (20 µL)
	1,000 tests

1. Introduction

Guanosine 3' : 5'-cyclic monophosphate (cGMP), a secondary messenger in signal transduction pathways, is produced by guanylate cyclase from GTP (1). cGMP mediates signalling in several physiological responses such as smooth muscle relaxation, penile erection, phototransduction, vascular tone, platelet aggregation, kidney function and inflammatory response.



cGMP is produced either by particulate guanylate cyclase upon binding of natriuretic peptides, guanylin or heat-stable enterotoxin to the extracellular domain of the molecule, or by nitric oxide (NO) which on penetrating into the cytosol, stimulates the soluble guanylate cyclase. Intracellular cGMP regulates cellular physiology by activating protein kinase G (PKG), directly gating specific ion channels, or altering intracellular cyclic nucleotide concentrations through the modulation of phosphodiesterase activity.

Phosphodiesterase regulates intracellular level of cGMP by cleaving the phosphodiester bond between the phosphorus and the oxygen atoms at the 3'-position.

2. Assay description and intended use

This assay is intended to be used for the quantitative determination of cGMP in buffered solution or cell-culture supernatants. The assay is based on the competition between sample cGMP and d2 labelled cGMP for binding to a Cryptate labelled antibody.

When mixed in the microtiter wells, sample cGMP and d2 conjugated cGMP will compete for binding to a limited number of sites on Cryptate conjugated anti-cGMP antibodies. As a direct consequence, the more cGMP contained in the sample, the less fluorescent signal will be seen.

HTRF® specific signal (i.e. energy transfer) is inversely proportional to the concentration of cGMP in the sample or standard.

3. Reagent preparation

3.1. Supplied reagents and preparation

Allow the reagents to warm up to room temperature for at least 30 minutes before reconstitution.

Supplied reagents	Reagent reconstitution (stock solutions) *	Working solutions**
anti cGMP-Cryptate 1 vial, lyophilized	Reconstitute each vial with 1 mL of distilled water. Mix gently.	⇒ For each vial dilute 1 volume of reconstituted reagent in 4 volumes of conjugate & lysis buffer (e.g. for 500 wells: 0.5 mL of reconstituted reagent + 2 mL of Conjugate & lysis buffer).
cGMP-d2 1 vial, lyophilized		
cGMP calibrator. Concentrated free cGMP. 1 vial, lyophilized	⇒ See indications on label for reconstitution volume.	⇒ See calibration curve preparation for further dilution
cGMP control. Free cGMP assay control. 1 vial, lyophilized	⇒ Mix gently after reconstitution.	⇒ To be used directly after reconstitution

Supplied reagents (continued)	Reagent reconstitution (Stock solutions)
cAMP & cGMP conjugates & lysis buffer (ref. 62CL1FDD) 50 mM Phosphate buffer, pH 7.0, 1M KF, 1.25 % Triton X100	1 vial See volume on the label
Diluent 50 mM Phosphate buffer, pH 7.0, 0.2 % BSA, 0.02 % NaN ₃ , preservatives	1 vial of 20 mL

Note : Supplementary cGMP calibrator (ref 62GMPCDA), cGMP control (ref 62GMPTDA) and diluent (ref 62DL1DDD) can be obtained separately on request.

*All lyophilized reagents must be reconstituted with distilled water.

**Conjugate working solutions must be prepared in individual vials and dispensed separately.

Precaution : HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the d2 and Cryptate-conjugates will impair the assay's quality.

Do not mix reagent from different batches or kits.

3.2. Reagent storage and stability

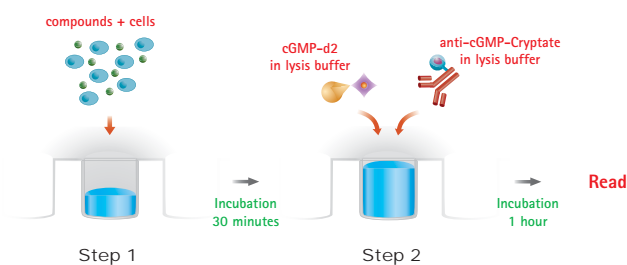
	Storage	Stability
Supplied reagents	4°C until reconstitution	Until expiry date indicated on the labels
Stock solutions	4°C	1 week
	frozen (-20°C)	Until expiry date indicated on the labels May be frozen and thawed twice
Working solutions of conjugates	4°C	1 week
	frozen (-20°C)	May be frozen and thawed twice

4. Assay protocol

The cGMP HTRF® assay is a four-dispensing step homogeneous time-resolved fluorescence assay in which free cGMP competes with an anti-cGMP-Cryptate / cGMP-d2 conjugate system.

Cell-based assay protocol includes 2 main steps :

1. Cell and compound dispensing, followed by incubation
2. Detection with HTRF reagents diluted in lysis buffer



4.1. Calibration curve preparation

Follow the dilution sequence shown in the following table to draw up the calibration curve. Dilution must be carried out with the diluent (or freshly made PO4 50mM, BSA 0.2% pH 7).

Calibrator	Preparation	cGMP working concentration (nM)	cGMP final concentration in assay (nM)
Cal 6	100 µL cGMP calibrator stock solution + 400 µL diluent	2,000	500
Cal 5	50 µL Cal 6 + 150 µL diluent	500	125
Cal 4	50 µL Cal 5 + 150 µL diluent	125	31.25
Cal 3	50 µL Cal 4 + 150 µL diluent	31.25	7.81
Cal 2	50 µL Cal 3 + 150 µL diluent	7.81	1.95
Cal 1	50 µL Cal 2 + 150 µL diluent	1.95	0.49
Cal 0 (Positive control)	200 µL diluent	0	0

4.2. Assay protocol for 384-well low volume plate (20µL final)

- ① Generation of the calibration curve

Negative control	Calibration curve	Assay control
10 µL diluent	5 µL calibrator	5 µL assay control
	5 µL diluent	5 µL diluent
5 µL conjugate & lysis buffer	5 µL cGMP-d2	5 µL cGMP-d2
5 µL anti cGMP-Cryptate	5 µL anti cGMP-Cryptate	5 µL anti cGMP-Cryptate

The assay control validates the accuracy of the calibration curve. The concentration deduced from the delta F obtained should fall into the concentration range indicated on the label of the vial.

② Cell based assay

Cell negative control	Cell condition
5 µL cells	5 µL cells
5 µL compound buffer	5 µL test compound
Cell stimulation (e.g. 30 minutes)	Compound incubation
5 µL conjugate & lysis buffer	5 µL cGMP-d2
5 µL anti cGMP-Cryptate	5 µL anti cGMP-Cryptate

- ③ Seal the plate and leave to incubate at room temperature for 1 hour.
- ④ Remove the plate sealer and read on PHERAstar Plus® (BMG-Labtech) or equivalent time-resolved fluorescence reader.

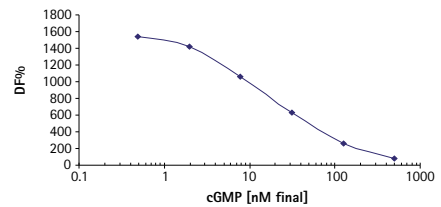
4.3. Data reduction

Example of a calibration curve :

	A (665nm)	B (620nm)	Ratio (1)	Mean Ratio (2)	CV % (3)	Delta F % (4)
Negative control	2,177 2,192	55,710 55,580	391 394	393	0.65	
[cGMP] nM final						
0	35,140	52,346	6,713	6,887	3.57	1,654
Positive control	37,167	52,637	7,061			
0.49	33,660 33,283	52,016 52,203	6,471 6,376	6,423	1.05	1,536
1.95	31,112 31,377	51,807 52,932	6,005 5,928	5,967	0.92	1,420
7.81	24,437 24,658	53,225 54,227	4,591 4,547	4,569	0.68	1,064
31.25	15,950 15,324	54,845 54,681	2,908 2,802	2,855	2.62	627
125	8,062 7,858	57,160 55,506	1,410 1,416	1,413	0.26	260
500	4,138 4,156	57,325 58,380	722 712	717	0.98	83
cGMP control	17,168 17,725	54,927 55,936	3,126 3,169	3,147	0.97	702

1. Ratio = $\frac{A_{665nm}}{B_{620nm}} \times 10^4$
2. Mean Ratio = $\frac{\sum \text{ratios}}{2}$
3. CV = $\frac{\text{Std deviation}}{\text{Mean ratio}} \times 100$
4. Delta F = $\frac{\text{Calibrator or sample Ratio} - \text{Ratio}_{neg}}{\text{Ratio}_{neg}} \times 100$

(Ratio_{neg} = negative control)



Delta F obtained for samples can be reported on the calibration curve to deduce respective cGMP concentration.

4.4. Assay characteristics

Analyte	% cross-reactivity
cGMP	100
GMP	0.003
GDP	0.001
GTP	0.003
cAMP	<0.001
AMP	<0.001
ATP	<0.001

	cGMP working concentration (nM)	cGMP final concentration in assay (nM)
Detection limit	2.8	0.7
EC ₅₀	84	21

EC₅₀ is the cGMP concentration which allows the displacement of 50% of binding.

5. Assay flexibility and miniaturization

When used as suggested, the kit will provide sufficient reagents for 1,000 tests using a 384- well low volume plate in 20 μL final assay volume (HTRF® packaged basis).

To move to other plate formats (96 half-well or 1536-well) and final volumes (100 μL to less than 10 μL), the volume of each assay component is simply proportionally adjusted in order to maintain the reagent concentrations as for the 20 μL final assay volume. For instance, in the case of the 1536-well format in 10 μL final volume, half as much material per well is used, thereby allowing 2,000 tests to be run. The performances of the HTRF® assay remain the same whatever the level of miniaturization.

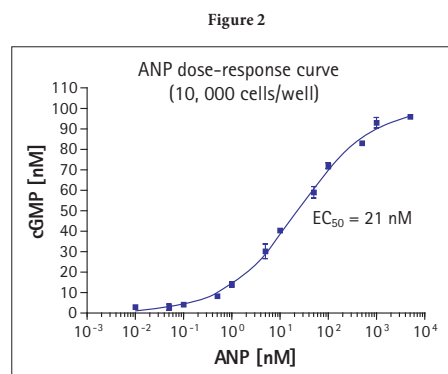
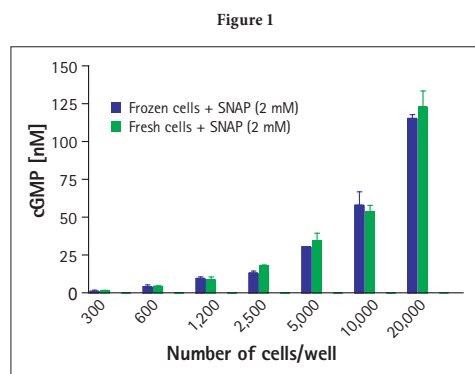
Assay components	Volume proportion	Assay format		
		1536-well (10 μL)	384-well low volume (20 μL)	96 half-well (100 μL)
Calibrator or cells	1 volume	2.5 μL	5 μL	25 μL
Diluent or test compound	1 volume	2.5 μL	5 μL	25 μL
d2 conjugate	1 volume	2.5 μL	5 μL	25 μL
Cryptate conjugate	1 volume	2.5 μL	5 μL	25 μL
		2,000 tests	1,000 tests	200 tests

Plate references : 96 half-well plate (Costar # 3694 or equivalent), 384-well low volume plate (Greiner # 784076), 1536-well (Greiner # 782086)

6. Cell density optimization

The cell density optimization is a key step in cyclic GMP kit use. Typically, the level of cGMP produced by cells must fall within the linear range of the calibration curve.

The optimization consists of testing a wide range of cell concentrations (e.g. between 300 and 20,104 cells per well), in the presence or absence of a direct activator of the cell Guanylate Cyclase (GC) enzyme, such as NO donor S-nitroso-N-acetylpenicil amine (SNAP) for soluble GC or Atrial Natriuretic Peptide (ANP) for the particulate GC. The addition of a phosphodiesterase inhibitor in cell dilution buffer is absolutely necessary (e.g. IBMX) in order to prevent cGMP degradation. The optimum cell density is the number of cells per well which leads to the highest signal amplitude obtained between the inactivated state (basal level of cGMP produced by cells) and the activated condition. The example shown below gives data generated on RFL-6 rat lung fibroblast using either SNAP (fig. 1) or ANP (fig. 2).



IMPORTANT :

- All lyophilized reagents must be reconstituted with distilled water.
- Proportional downsizing of assay components should be strictly applied for miniaturization (see assay flexibility and miniaturization section).
- Never pre-mix the two HTRF® conjugates before dispensing, in order to avoid a pre-kinetic equilibrium between the two components.
- Readout should only be carried out with HTRF® compatible instruments.