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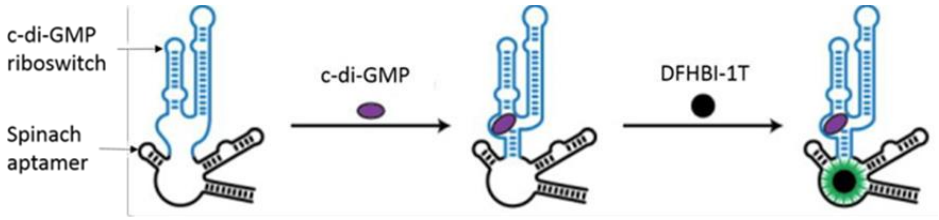
Cyclic-di-GMP Assay Kit

10/20

(Catalog # K1483-100; 100 assays; Store at -20°C)

I. Introduction:

BioVision's Cyclic-di-Guanosine Monophosphate (cyclic-di-GMP) Assay Kit is a simple, rapid, highly selective assay to measure cyclic-di-GMP levels in cells. This assay can be used to detect cyclic-di-GMP in any biochemical or enzymatic reaction that produces cyclic-di-GMP or in cell-based applications to monitor intracellular cyclic-di-GMP concentrations. In this assay, the evolution of the fluorescent signal is dependent on the initial binding of cyclic-di-GMP to the cyclic-di-GMP riboswitch in the sensor. This results in the stabilization of the Spinach™ aptamer, which in turn binds to the DFHBI-1T Fluorophore to produce a stable fluorescence, that can be measured at Ex/Em = 482/505 nm. The assay is easy to use and is homogenous. The end user needs to simply incubate the samples with the provided assay reagents for 30 min followed by reading the samples on a fluorescence plate reader at Ex/Em = 482/505 nm.



II. Applications:

- Cell-based applications to monitor intracellular cyclic-di-GMP concentrations in bacteria.
- To detect cyclic-di-GMP in any biochemical or enzymatic reaction that produces cyclic-di-GMP.

III. Sample Types:

- Bacterial cells
- Biochemical samples

IV. Key Features:

- Simple, easy to use
- Highly selective for cyclic-di-GMP with no interference from common counter ligands such as GMP, c-AMP-GMP, pGpG etc.
- Quick response and good signal stability
- Broad dynamic range (sensitive even at 50 nM of cyclic-di-GMP)
- Compatible with cell based assays

V. Kit Contents:

Components	K1483-100	Part Number
Cyclic di-GMP Assay Buffer (4X)	10 ml	K1483-100-1
Bacterial Compatibility Reagent (4X)	10 ml	K1483-100-2
c-di-GMP Sensor	1 vial	K1483-100-3
c-di-GMP	1 vial	K1483-100-4
DFHBI-1T Fluorophore	1 vial	K1483-100-5
RNase-Free Water	15 ml	K1483-100-6

VI. User Supplied Reagents and Equipment:

- DMSO
- RNase-free microfuge tubes
- Fluorescence plate reader (Ex/Em = 482/505 nm)
- 96-well black flat bottom plates

VII. Storage Conditions and Reagent Preparation:

The kit is shipped at room temperature (RT). Upon receipt, store the reagents at the recommended storage conditions. Read the entire protocol before performing the assay.

- **c-di-GMP Assay Buffer (4X)** and **Bacterial Compatibility Reagent (4X):** Store at RT.
- **c-di-GMP Sensor:** Reconstitute the vial in 1.2 ml RNase-Free Water to prepare c-di-GMP Sensor stock solution (20X or 6.5 µM). Pipette up and down to mix well. Divide into aliquots in RNase-free tubes and **store at -80°C**. Avoid repeated freeze/thaw cycles.
- **c-di-GMP:** Reconstitute the vial in 1.0 ml RNase-Free Water to prepare c-di-GMP stock solution (1000X or 10 µM). Pipette up and down to mix well. Divide into aliquots in RNase-free tubes and store at -20°C. Avoid repeated freeze/thaw cycles.
- **DFHBI-1T Fluorophore:** Reconstitute the vial in 50 µl DMSO to prepare DFHBI-1T Fluorophore stock solution (1000X or 10 mM). Divide into aliquots in RNase-free tubes and store at -20°C. Avoid repeated freeze/thaw cycles.

VIII. Cyclic-di-GMP Assay Protocol:

A. 10X cyclic-di-GMP Standard Preparation: Prepare the 10X Standards in RNase-free microfuge tubes as described in the table below. Mix the Standards thoroughly.

10X Standards	Volume of cyclic-di-GMP	RNase-Free Water	1X cyclic-di-GMP Conc (nM, pg/µl)
Standard 1	100 µl of 1000X cyclic-di-GMP Stock	0 µl	1000 nM or 690.09 pg/µl
Standard 2	30 µl of Standard 1	10 µl	750 nM or 517.57 pg/µl
Standard 3	20 µl of Standard 1	20 µl	500 nM or 345.05 pg/µl
Standard 4	10 µl of Standard 1	20 µl	250 nM or 172.52 pg/µl
Standard 5	4 µl of Standard 1	36 µl	100 nM or 138.02 pg/µl
Standard 6	4 µl of Standard 3	36 µl	50 nM or 34.50 pg/µl
Standard 7	4 µl of Standard 4	36 µl	25 nM or 17.25 pg/µl
Standard 8	0 µl	40 µl	0 nM or pg/µl

Notes:
The 10X Standard working stocks can be used to derive the Standard Curve and to estimate cyclic-di-GMP concentrations in biochemical samples and in cell-based assays. The molecular mass of c-di-GMP = 690.40 Da

B. Prepare 10X DFHBI-1T Fluorophore solution by diluting the 1000X Fluorophore Stock at 1:100 dilution with RNase-free water. Prepare enough volume of 10X DFHBI-1T Fluorophore for the assay. Discard the unused 10X Fluorophore solution after each use.

Biochemical Assay Protocol:

1. **Addition of Standards:** Add 20 µl of 10X Standards (Standards 1-8) to the appropriate Standard wells in a 96-well black plate. Add other reagents as mentioned below in the table as **Standard Well** set up.
2. **Addition of Unknown Sample(s):** Up to 120 µl of the Unknown Sample containing cyclic-di-GMP can be tested using the assay. If the sample volume is below 120 µl, adjust the volume to 120 µl/well with RNase-Free water. Add other reagents as mentioned below in the table as **Unknown Sample Well** set up.

Name of the Reagent	Standard Well	Unknown Sample(s) Well
	10 X Standards	Unknown Sample(s)
10X Standards	20 µl	--
Unknown Sample(s)	--	Up to 120 µl
RNase-Free Water	100 µl	--
cyclic-di-GMP Assay Buffer (4X)	50 µl	50 µl
10X Fluorophore solution	20 µl	20 µl
20X c-di-GMP Sensor	10 µl	10 µl
Total	**200 µl**	**200 µl**

3. Incubate the plate for 30 min at RT in a dark place. The incubation time can be extended up to 24 hr without affecting the assay.
4. Read the plate in a fluorescence plate reader at Ex/Em = 482/505 nm.

Cell-Based Assay Protocol:

1. Addition of Standards: Add 20 µl of 10X cyclic-di-GMP Standards (Standards 1-8) to the appropriate Standard wells in a 96-well black plate. Add up to 50 µl of appropriate bacterial culture media to each of the Standard wells and adjust the total volume/well to 70 µl with RNase-free water. Add other reagents as mentioned below in the table as **Standard Well** set up.

2. Addition of Unknown Sample(s): Up to 50 µl of the bacteria in culture media can be tested using the cyclic-di-GMP assay. Adjust the volume to 70 µl/well with RNase-free water. Add other reagents as mentioned below in the table as **Unknown Sample Well** set up.

	Standard Well	Unknown Sample(s) Well
Name of the Reagent	**10X Standards**	**Bacterial Culture**
10X Standards	20 µl	--
Bacterial Culture	--	Up to 50 µl
Culture Media	Up to 50 µl	--
RNase-Free Water	--	20 µl
cyclic-di-GMP Assay Buffer (4X)	50 µl	50 µl
4X Bacterial Compatibility Reagent	50 µl	50 µl
10X Fluorophore solution	20 µl	20 µl
20X c-di-GMP Sensor	10 µl	10 µl
Total	**200 µl**	**200 µl**

4. Incubate the plate for 30 min at RT in a dark place. The incubation time can be extended up to 24 hr without affecting the assay.
5. Read the plate in a fluorescence plate reader at Ex/Em = 482/505 nm.

IX. Determination of Cyclic-di-GMP Concentration:

1. Plot the cyclic-di-GMP Standard Curve: Plot the fluorescence values of the Standards as a function of their final cyclic-di-GMP concentration (1X) from Table 1. Fit a straight line (y = mx + b) connecting the data points. Calculate the slope (m) and Y intercept (b).

2. Estimating the cyclic-di-GMP Concentration: Determine the fluorescence intensity (RFU) of the Unknown Samples. Calculate the c-di-GMP concentrations in the Unknown Samples using the following equation:

X (Conc. in Unknown Sample, pg/µl) = (Y (RFU of Unknown Sample) – b) / m.

Appropriate sample dilution factors must be multiplied to get the c-di-GMP concentrations (pg/µl) in the undiluted samples.

IX. Cyclic-di-GMP Estimation in Bacteria: The c-di-GMP assay can be used to measure the intracellular cyclic-di-GMP concentrations in bacteria in a homogenous format without pelleting, lysis, or wash steps. Simply supplement the assay buffer containing bacterial culture with BC reagent, add sensors and fluorophores, incubate for 30 min, and read on a fluorescence reader. *E.coli* cells expressing wild type WspR (a diguanylate cyclase from Pseudomonas aeruginosa) were treated with nitrofurazone (antibiotic), dimethyl formamide/DMF (carrier), or untreated (control) (Figure 2 A). In addition, the bacteria were also plated at varying cell densities to simulate varying c-di-GMP levels (Figure 2B). Cyclic-di-GMP concentrations in bacterial cells growing in a 96-well plate were measured without any pelleting, washes, lysis, or organic extraction.

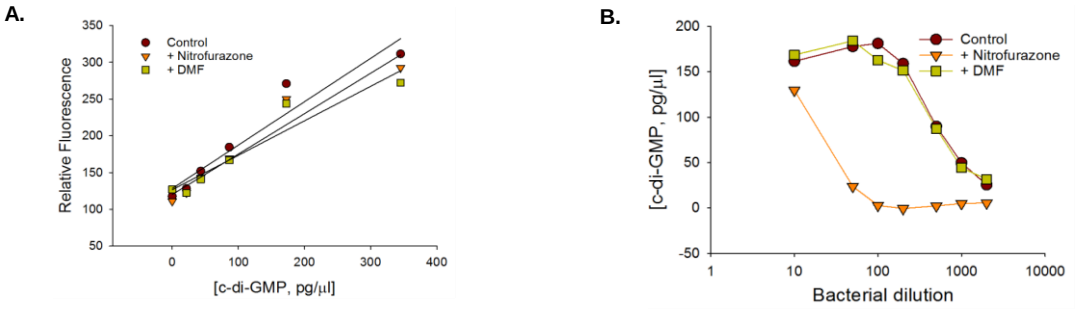
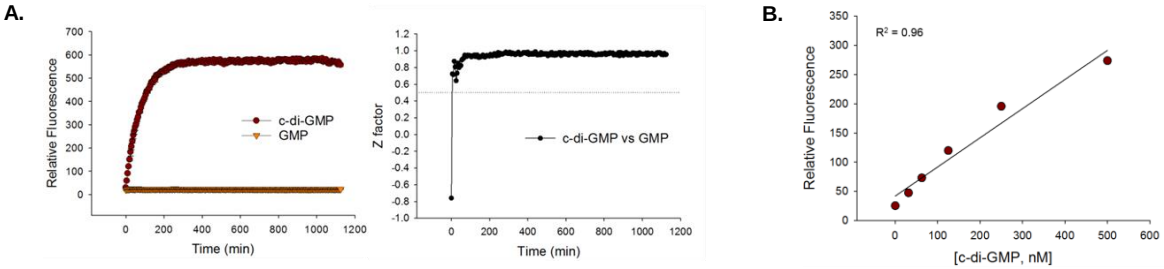
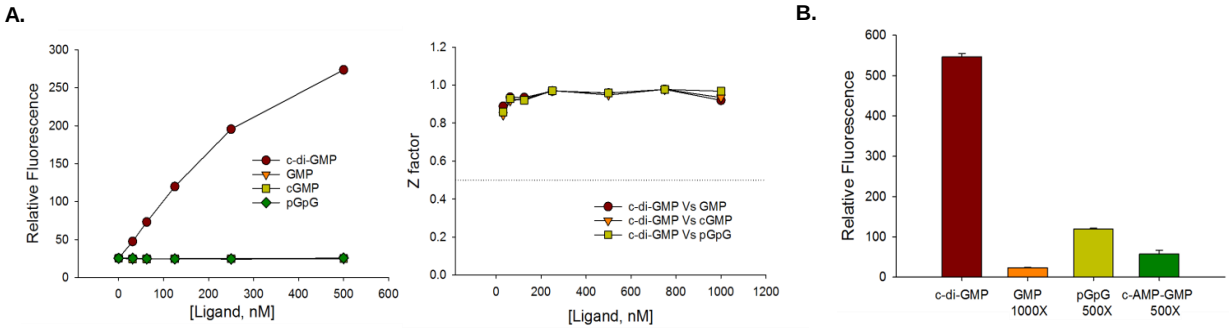


Figure 2. A. Cyclic-di-GMP Standard Curve. B. Cyclic-di-GMP estimates in WT WspR cells. Presence of antibiotic or carrier did not affect the performance of the c-di-GMP assay. The cyclic-di-GMP assay shows the changes in c-di-GMP concentrations in bacteria in response to antibiotic treatment and varying bacterial cell densities.

X. Quick Response, Good Signal Stability and Broad Dynamic Range: The Cyclic-di-GMP sensor produces signals rapidly upon cyclic-di-GMP detection, and thus allows fast measurement. The fluorescent signal is stable over time and thus allows batch-mode processing of samples. Excellent HTS parameters are attained as early as 30 min of assay setup and remain high for a prolonged period of time (Figure 3A). The assay is sensitive even at 50 nM of cyclic-di-GMP and has a broad dynamic range (Figure 3B).



XI. Selectivity: The c-di-GMP sensor is highly selective for c-di-GMP with no interference from common counter ligands (Figure 4A). The assay achieves excellent HTS assay parameter (Z factor >0.9) even when compared with 500 - 1,000-fold excess of counter ligands such as GMP, pGpG, and c-AMP-GMP (Figure 4B).



XI. Related Products:

cGMP Direct Immunoassay Kit (Colorimetric) (Cat# K372)
cGMP Antibody (Cat# 3568)
cGMP ELISA Kit (Cat# E4717)
Anti-cGMP Antibody (2F11E10) (Cat# A1285)

FOR RESEARCH USE ONLY! Not to be used on humans.