Cyclic-di-GMP Assay Kit

Catalog Number: 200-100

(Manual rev. 2.0)

For in vitro research use only

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1.0 REAGENTS

1.1 Supplied in the Kit

Reagent	Amount	Storage
c-di-GMP Sensor	400 µg	-80°C
c-di-GMP	6.9 µg	-20°C
1,000X DFHBI-1T Fluorophore	50 µL	-20°C
4X c-di-GMP Assay (CA) Buffer	10 mL	25°C
4X Bacterial Compatibility (BC) Reagent	10 mL	25°C
RNase-Free Water	15 mL	25°C

This kit is shipped at room temperature. Upon receipt, store reagents at the recommended storage conditions. Reconstituted sensor should be aliquot into RNase-free tubes and store at -80°C. Avoid repeated freeze/thaw.

1.2 Reconstitution

Reagent	Resuspension	Final Concentration
c-di-GMP Sensor Stock (20X)	1.2 mL RNase-Free H_2O	6.5 µM
c-di-GMP Stock	1 mL RNase-Free H_20	10 µM

IMPORTANT: Make sure to spin down each reagent tubes before resuspension!

1.3 Supplied by the User

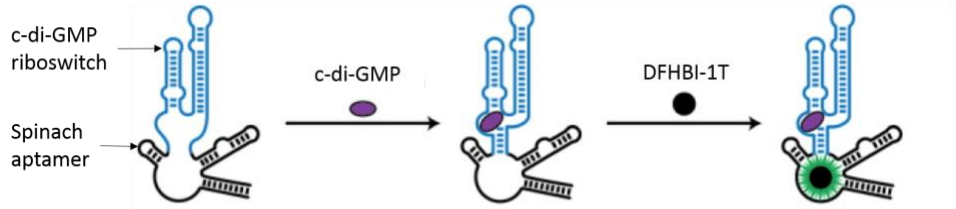
1. RNase-free microfuge tubes
2. Fluorescence plate reader with FITC filter set or capable of excitation at 482nm and emission at 505nm.
3. Black flat bottom assay plates suitable for fluorescence reading

2.0 ASSAY DESCRIPTION

The cyclic-di-guanosine monophosphate (c-di-GMP) assay is a simple mix-and-read, highly selective, HTS-ready assay to measure c-di-GMP levels in cells. This homogenous assay can be used to detect c-di-GMP in any biochemical or enzymatic reaction that produces c-di-GMP or in cell-based applications to monitor intracellular c-di-GMP concentrations.

2.1 Assay Principle

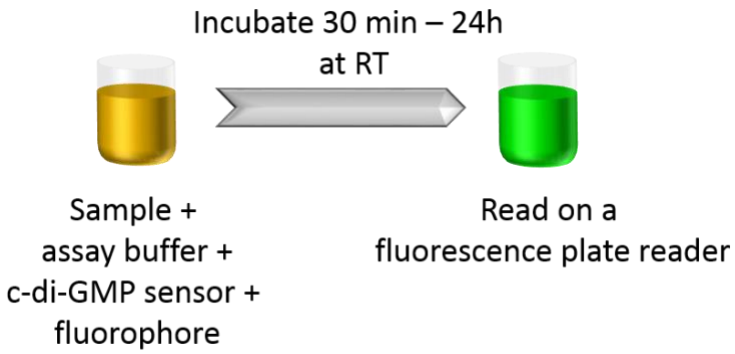
The evolution of the fluorescent signal is dependent on the initial binding of c-di-GMP to the c-di-GMP riboswitch in the sensor. This results in the stabilization of the Spinach™ aptamer, which in turn binds DFHBI-1T to produce fluorescence. The fluorescence can be measured using GFP/FITC filter sets or with excitation at 482nm and emission at 505nm.



Kellenberger et al JACS, 2013

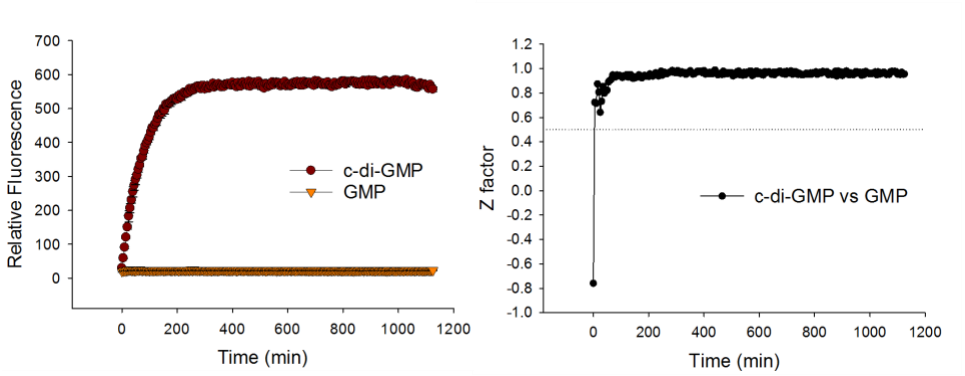
2.2 Ease of Use

The c-di-GMP assay is easy-to-use and homogenous. Simply incubate your samples with the provided assay reagents for 30 min and read the samples on a fluorescence plate reader with FITC/GFP filter or excitation wavelength 482nm and emission wavelength 505nm.



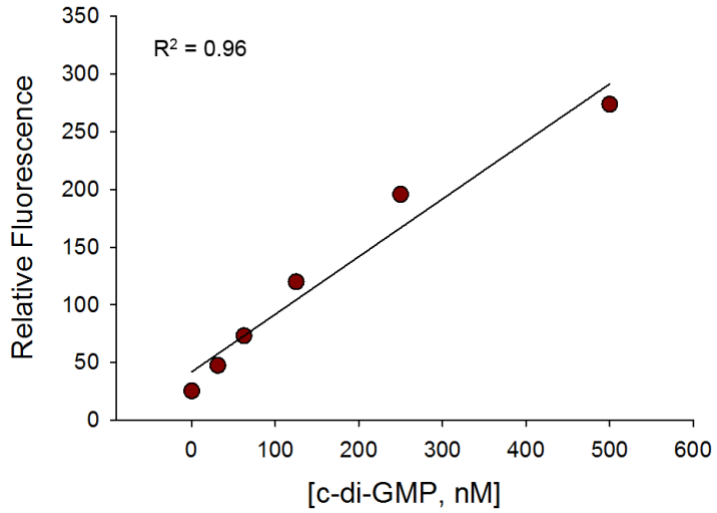
2.3 Quick Response and Good Signal Stability

The c-di-GMP sensor produces signals rapidly upon c-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.



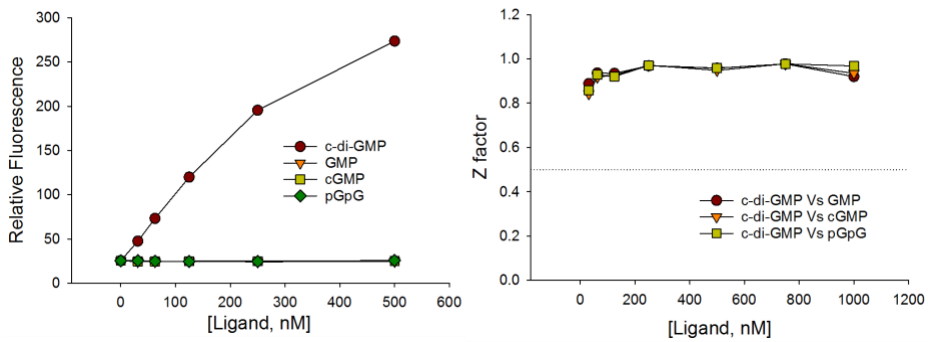
2.4 Broad Dynamic Range

The assay is sensitive even at 50nM of c-di-GMP and has a broad dynamic range.

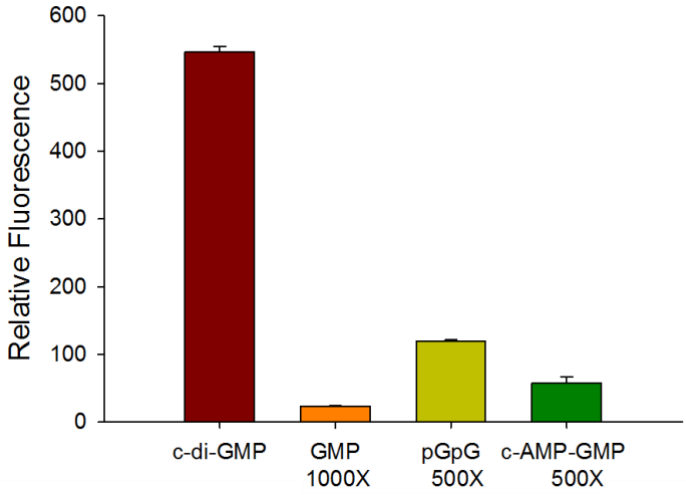


2.5 Selectivity

The c-di-GMP sensor is highly selective for c-di-GMP with no interference from common counter ligands.

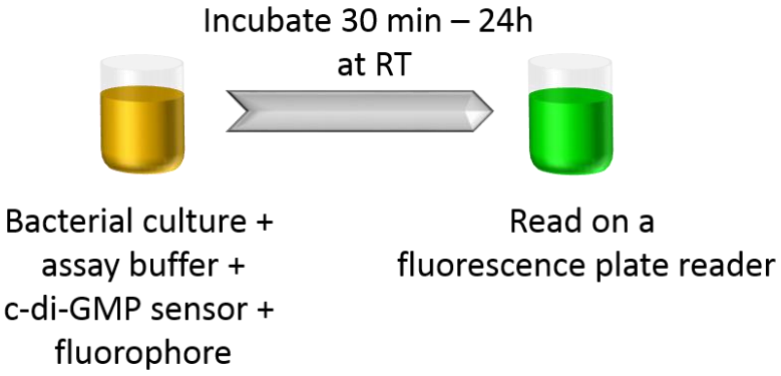


The assay achieves excellent HTS assay parameter (Z factor >0.9) even when compared with 500 - 1,000-fold excess of counter ligands such as GMP, pGpG, and c-AMP-GMP.



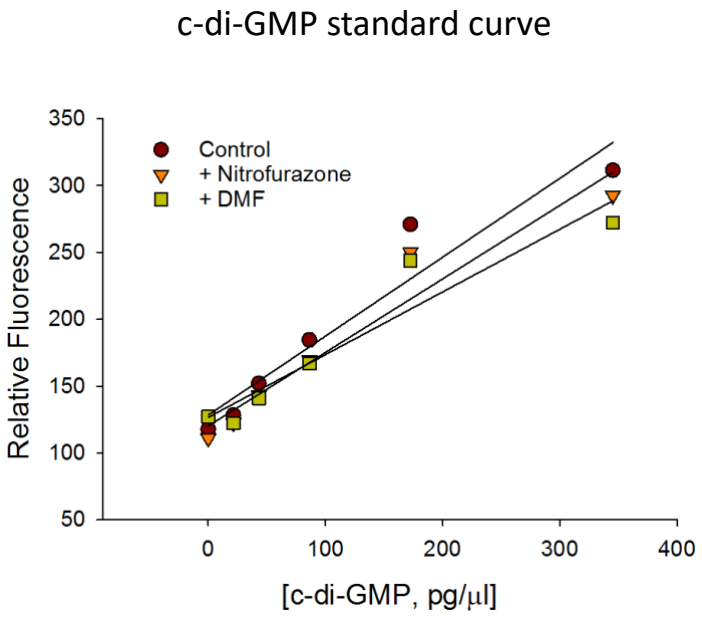
2.6 Compatibility with Cell-Based Assays

The c-di-GMP assay can be used to measure intracellular c-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.

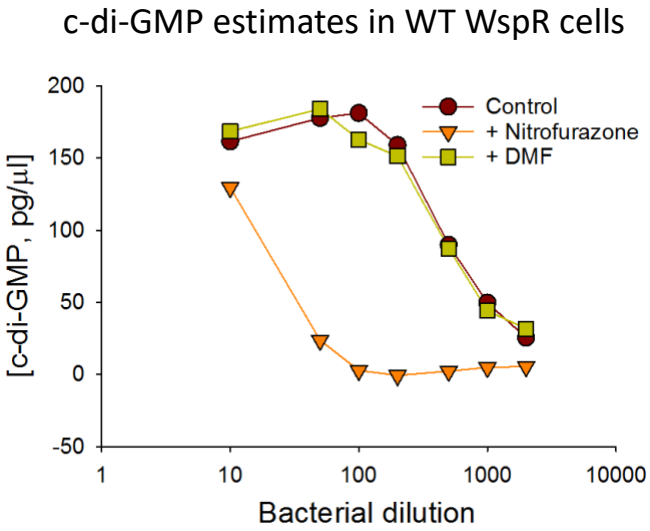


2.7 c-di-GMP Estimation in Bacteria

E.coli cells expressing wild type WspR (a diguanylate cyclase from *Pseudomonas aeruginosa*) were treated with nitrofurazone (antibiotic), dimethyl formamide/DMF (carrier), or left untreated (control). In addition, the bacteria were also plated at varying cell densities to simulate varying c-di-GMP levels. Cyclic-di-GMP concentrations in bacterial cells growing in a 96-well plate were measured without any pelleting, washes, lysis, or organic extraction.



Presence of antibiotic or carrier did not affect the performance of the c-di-GMP assay.



The c-di-GMP assay shows the changes in c-di-GMP concentrations in bacteria in response to antibiotic treatment and varying bacterial cell densities.

3.0 ASSAY PROTOCOL

The c-di-GMP assay can be used to determine c-di-GMP concentrations in biochemical assays and in cell-based applications.

3.1 Preparation of Reagents

3.1.1. *10X c-di-GMP Standards:* Prepare the 10X standards as described in Table 1 in RNase-free microfuge tubes. Mix the standards thoroughly.

10X Standards	Volume of c-di-GMP	Volume of RNase-Free Water	Final Assay Concentration (nM, pg/µl)
Standard 1	100 µl of c-di-GMP Stock	0 µl	1,000, 690.09
Standard 2	30 µl of Standard 1	10 µl	750, 517.57
Standard 3	20 µl of Standard 1	20 µl	500, 345.05
Standard 4	10 µl of Standard 1	30 µl	250, 172.52
Standard 5	4 µl of Standard 1	36 µl	100, 69.01
Standard 6	4 µl of Standard 3	36 µl	50, 34.50
Standard 7	4 µl of Standard 4	36 µl	25, 17.25
Standard 8	0 µl	40 µl	0

Table 1: Preparation of c-di-GMP 10X Standard solutions.
Molecular mass of c-di-GMP = 690.40 Da

The 10X Standard working stocks can be used to derive the standard curve and to estimate c-di-GMP concentrations in biochemical samples and in cell-based assays.

3.1.2. *10X Fluorophore Solution:* Dilute the 1,000X Fluorophore Stock 1:100 with RNase-free water to make 10X Fluorophore Solution. Prepare only sufficient volume of 10X fluorophore for the assay. Discard the unused 10X Fluorophore Solution after the assay.

3.2 Biochemical Assay Protocol

3.2.1. *Standard Samples:* Add 20 µl of the 10X c-di-GMP Standards (Standard 1 - 8) to the appropriate standard wells in a black bottom 96-well assay plate. Add 100 µl of RNase-free water to each of the standard wells (Refer to *Table 2*).

3.2.2. *Unknown Samples:* Up to 120 µl of the unknown sample containing c-di-GMP can be tested using the c-di-GMP assay. If the sample volume is below 120ul, make up the volume in the well to 120 µl with RNase-free water (Refer *Table 3*). *Do not use culture media to make up the volume as it could increase background specific fluorescence.*

3.2.3. *Reagent Addition:* Add 50 µl of 4X c-di-GMP assay (CA) buffer, 20 µl of 10X fluorophore, and 10 µl of sensor to the each of the wells (*standard and unknown samples*).

3.2.4. *Incubation:* Incubate the plate for 30 minutes at room temperature in a dark place. The incubation time can be extended up to 24h without affecting the performance of the assay.

3.2.5. *Reading:* Read the plate in a fluorescence plate reader with a GFP/FITC filter set or with excitation set at 482nm and emission at 505nm. (*In some instruments, excitation set at 469nm and emission at 501nm generated lower background signals. Testing each wavelength sets with the c-di-GMP standard samples is recommended to determine the optimal experimental wavelengths.)*

Name of Reagent	Volume/Well
10X Standards	20 µl
RNase-Free Water	100 µl
4X c-di-GMP Assay (CA) Buffer	50 µl
10X Fluorophore	20 µl
20X c-di-GMP Sensor	10 µl
Total	**200 µl**

Table 2: Standard sample assay setup

Name of Reagent	Volume/Well
Sample[3.2.2]	Up to 120 µl
RNase-Free Water	Make up sample volume to 120 µl
4X c-di-GMP Assay (CA) Buffer	50 µl
10X Fluorophore	20 µl
20X c-di-GMP Sensor	10 µl
Total	**200 µl**

Table 3: Unknown sample assay setup

3.3 Cell-Based Assay Protocol

3.3.1. *Standard Samples:* Add 20 µl of the 10X c-di-GMP standards (Standard 1 - 8) to the appropriate standard wells in a black bottom 96-well assay plate. Add up to 5 µl of appropriate bacterial culture media to each of the standard wells (Refer *Table 4*). Make up the total volume to 50 µl with RNase-free water. *Do not use culture media to make up the volume as it could increase background specific fluorescence.*

3.3.2. *Unknown Samples:* Dilute the culture media with bacteria at least 1:10 in RNase-free water. Up to 50 µl of the diluted bacteria in culture media can be tested using the c-di-GMP assay. Make up the volume in the well to 70 µl with RNase-free water (Refer *Table 5*). *Do not use culture media to make up the volume.*

3.3.3. *Reagent Addition:* Add 50 µl of 4X c-di-GMP assay (CA) buffer, 50ul of bacterial compatibility (BC) reagent, 20 µl of 10X fluorophore, and 10 µl of sensor to the each of the wells (*standard and unknown samples*).

3.3.4. *Incubation:* Incubate the plate for 30 minutes at room temperature in a dark place. The incubation time can be extended up to 24h without affecting the performance of the assay.

3.3.5. *Reading:* Read the plate in a fluorescence plate reader with a GFP/FITC filter set or with excitation set at 482nm and emission at 505nm. (*In some instruments, excitation set at 469nm and emission at 501nm generated lower background signals. Testing each wavelength sets with the c-di-GMP standard samples is recommended to determine the optimal experimental wavelengths.)*

Name of Reagent	Volume/Well
10X Standards	20 µl
Culture Media	Up to 5 µl
RNase-Free Water	Make up volume to 50 µl
4X c-di-GMP Assay (CA) Buffer	50 µl
4X Bacterial Compatibility (BC) Reagent	50 µl
10X Fluorophore	20 µl
20X c-di-GMP Sensor	10 µl
Total	**200 µl**

Table 4: Bacterial culture standard sample assay setup

Name of Reagent	Volume/Well
Diluted Bacterial Culture[3.3.2]	Up to 50 µl
RNase-Free Water	Make up volume to 70 µl
4X c-di-GMP Assay (CA) Buffer	50 µl
4X Bacterial Compatibility (BC) Reagent	50 µl
10X Fluorophore	20 µl
20X c-di-GMP Sensor	10 µl
Total	**200 µl**

Table 5: Bacterial culture unknown sample assay setup

4.0 DETERMINATION OF CYCLIC-DI-GMP CONCENTRATION

4.1 Plot the Standard Curve

Plot the fluorescence values of the c-di-GMP standards as a function of their final c-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).

4.2 Estimating the c-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.

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