Application Note

Quantification of Cyclic Dinucleotides During Growth Phase in Three Bacterial Species Using ELISAs

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Key Features

- Measurement of 3'3'-cGAMP, cyclic di-GMP, and cyclic di-AMP in bacteria samples using Cayman's ELISAs.
- ELISA data provides accurate and reliable results over a wide range of dilutions.
- Bacterial cyclic dinucleotides (CDNs) can be measured in growth media and supernatants by ELISA without a cumbersome organic extraction.
- Bacterial supernatants contain significantly higher levels of CDNs compared to corresponding lysates.
- Different bacteria species show unique CDN profiles during growth phase in both lysates and supernatants.
- ELISAs provide a user-friendly and cost-effective method to quantify CDN concentrations in bacteria supernatants to better understand the role these second messengers play.

Introduction

Cyclic dinucleotides (CDNs) are secondary signaling messengers in both prokaryotic and eukaryotic organisms. They are produced by specific dinucleotide cyclases and degraded by phosphodiesterases.[1] In bacteria, CDNs regulate critical processes including metabolism, motility, biofilm formation, virulence, and survival.[2,3] One of the major hindrances in understanding the roles that CDNs play has been accurate quantification in biological samples. Due to the water-soluble nature of these compounds, LC-MS can be difficult, and purifications are cumbersome. To address these problems, we developed simple ELISA-based methods to quantify 3'3'-cGAMP, cyclic di-GMP, and cyclic di-AMP in samples. These assays provide researchers with rapid, cost-effective methods to quantitate CDNs and measure the effects under various biological conditions. Here we show data validating these ELISA methods and use them to quantify CDNs in lysates and supernatants from various bacterial genera including *Streptomyces*, *Pseudomonas*, and *Mycobacterium*.

Methods

Media

Pseudomonas aeruginosa and *Mycobacterium rhodochrous* were grown in TYGE media containing 5 g/L tryptone, 3 g/L yeast extract, 1 g/L glucose, and 1 g/L potassium phosphate (dibasic), pH 7.0. *Streptomyces cinnamoneus* was grown in ISP2 media containing 4 g/L yeast extract, 10 g/L malt extract, and 4 g/L glucose, pH 7.3.

Growth & Sampling

Seed cultures (100 ml) were inoculated with glycerol stocks and incubated for 24 hours at 28°C with shaking at 200 RPM. Growth cultures (1 L) were inoculated with seed cultures at 1% inoculum and incubated at 28°C with shaking at 200 RPM. For sampling, 50 ml of the culture was removed, OD_{600} measured, and centrifuged at 4,000 x g for 30 minutes. Supernatant was decanted, reserving 5 ml for analysis by ELISA, and the pellet stored at -80°C until processing. Culture samples were taken at 0, 12, 24, 48, 72, 96, 144, and 192 hours.

ELISA Sample Preparation

Cell counts of the bacterial pellets were determined using OD_{600} and lysed using B-PER™ Bacterial Protein Extraction Reagent (ThermoFisher Scientific) at a concentration of 10^{10} cells per ml of extraction reagent. Lysate protein was quantified by BCA. For Bligh and Dyer extraction, samples were diluted with water, followed by the addition of equal volumes of methanol and chloroform. The biphasic mixture was vortexed before being centrifuged at 16,100 x g for five minutes. The aqueous top layer was dried and reconstituted in water equivalent to the initial sample volume.

ELISAs

Lysates and supernatants were evaluated in Cayman's 3'3'-cGAMP ELISA Kit (Item No. 502130), Cyclic di-GMP ELISA Kit (Item No. 501780), and Cyclic di-AMP ELISA Kit (Item No. 501960) in duplicate with multiple dilutions of each sample according to the kit booklets. Only the sample dilutions that fell within the linear portion of the standard curve (20-80% B/B_0) were included in calculations. Where applicable, ELISA data was converted to pM from pg/ml. ELISA values were normalized to OD_{600} and adjusted for sampling volume.

Results

The growth media was evaluated in all three ELISAs with and without extraction showing a 70-90% efficiency with organic extraction (**Tables 1 and 2**). This data also indicates there are baseline levels of the CDNs in the growth media. These baseline levels are mainly due to the included yeast and malt supplements (data not shown).

	No Extraction	Organic Extraction	Extraction Efficiency
3'3'-cGAMP (pM)	6,190	5,690	91.9%
Cyclic di-GMP (pM)	4,422	3,651	82.6%
Cyclic di-AMP (pM)	10,103	7,774	76.9%

Table 1. Baseline CDN levels of the growth media used for *Pseudomonas aeruginosa* and *Mycobacterium rhodochrous*.
The media was either diluted directly in the assay buffer or extracted using the Bligh and Dyer method and assessed in the ELISA.

	No Extraction	Organic Extraction	Extraction Efficiency
3'3'-cGAMP (pM)	9,080	8,067	88.8%
Cyclic di-GMP (pM)	6,380	4,725	74.1%
Cyclic di-AMP (pM)	3,054	2,192	71.8%

Table 2. Baseline CDN levels of the growth media used for *Streptomyces cinnamoneus*.
The media was either diluted directly in the assay buffer or extracted using the Bligh and Dyer method and assessed in the ELISA.

Bacterial lysates and supernatants were measured at multiple dilutions (in duplicate) over the course of eight days for each CDN. With all bacterial species, the three ELISAs performed similarly, showing consistent calculated CDN levels independent of dilution. Average percent coefficient of variation (%CV) of multiple dilutions for each sample was less than 15%, indicating dependable and accurate ELISA results. **Tables 3 and 4** are examples of this, showing the linearity of multiple dilutions from *Pseudomonas aeruginosa* lysate and supernatant samples, respectively, in the 3'3'-cGAMP ELISA Kit.

Time (Days)	Dilution	3'3'-cGAMP (pM)	Average (pM)	%CV
0.5	4	450	455	1.5
	8	460		
1	4	523	522	0.3
	8	523		
	16	520		
2	4	422	423	3.0
	8	436		
	16	411		
3	8	1,259	1,215	5.1
	16	1,171		
4	4	494	510	3.3
	8	509		
	16	528		
5	4	604	588	2.4
	8	581		
	16	580		
6	2	368	371	2.8
	4	359		
	8	384		
	16	374		
7	4	609	641	3.8
	8	658		
	16	663		
	32	635		
8	8	789	801	2.2
	16	814		

Table 3. Linearity of multiple dilutions of *Pseudomonas aeruginosa* lysate samples in the 3'3'-cGAMP ELISA Kit.

Time (Days)	Dilution	3'3'-cGAMP (pM)	Average (pM)	%CV
1	80	46,212	47,583	6.8
	160	45,259		
	320	51,279		
2	40	49,841	46,369	7.0
	80	46,817		
	160	46,795		
	320	42,023		
3	80	72,878	74,058	4.0
	160	71,865		
	320	77,432		
4	40	51,635	47,847	8.0
	80	48,737		
	160	48,497		
	320	42,521		
5	40	52,051	55,385	4.3
	80	55,480		
	160	56,455		
	320	57,553		
6	40	75,339	72,159	4.7
	80	72,551		
	160	68,588		
7	40	132,946	136,552	1.8
	80	137,346		
	160	137,241		
	320	138,673		
8	80	88,370	83,578	6.5
	160	84,638		
	320	77,725		

Table 4. Linearity of multiple dilutions of *Pseudomonas aeruginosa* supernatant samples in the 3'3'-cGAMP ELISA Kit.

Interestingly, supernatant values were found to be several folds higher than lysate values. Each bacterial species presents its own unique CDN profile during growth phase. Day zero in the supernatant figures (**Figure 1B, -D, and -F**) indicate the baseline presence of CDNs found in the growth media. Fluctuations of each analyte can be observed over time, indicating either possible transport or modulation of the CDNs. Comparison of the supernatant figures with the lysate figures (**Figure 1A, -C, and –E**) for each bacteria species reveals related trends over the course of the growth phase for each CDN.

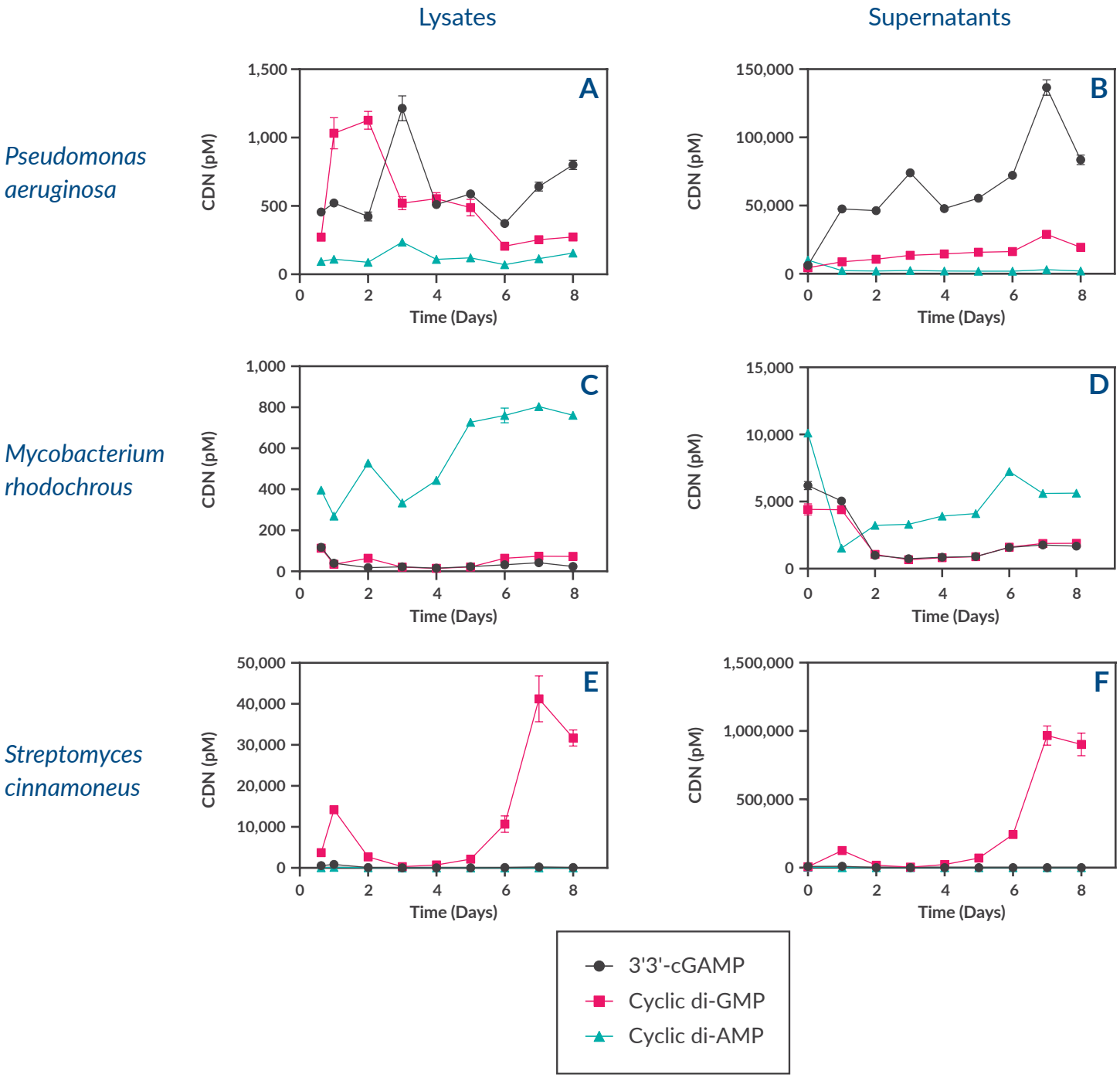

Figure 1. CDN composition in bacterial lysates and supernatants during growth phase. Error bars represent standard deviations obtained from multiple dilutions of each sample.

Conclusions

The use of ELISAs for measuring CDNs is an inexpensive and convenient method compared to the cost associated with mass spectrometry. In these experiments, we have demonstrated that it is not necessary to utilize complex organic extraction methods for isolating the CDN of interest before analyzing samples in an ELISA. The ELISA provides precise and reproducible data that is validated by consistent calculation of multiple dilutions and low coefficient of variation with minimal cross-reactivity (**Table S1 on page 7**).

Historically, researchers have mainly focused on CDN levels in bacterial lysates. Here we have presented interesting data hinting towards an extracellular component to the CDN story that has yet to be elucidated. Without the need for a complicated extraction or isolation procedure, bacterial supernatants prove to be a straightforward sample matrix to measure in the ELISA while also following similar trends seen in corresponding lysates.

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Cayman products used in this application note

Item No.	Product Name
502130	3'3'-cGAMP ELISA Kit
501780	Cyclic di-GMP ELISA Kit
501960	Cyclic di-AMP ELISA Kit

References

1. Chang, D., Whiteley, A.T., Bugda Gwilt, K., *et al*. Extracellular cyclic dinucleotides induce polarized responses in barrier epithelial cells by adenosine signaling. *Proc. Natl. Acad. Sci. USA* **117(44)**, 27502-27508 (2020).
2. Danilchanka, O. and Mekalanos, J.J. Cyclic dinucleotides and the innate immune response. *Cell* **154(5)**, 962-970 (2013).
3. Wilburn, K.M., Blaylock, J.B., Metcalfe, K.C., *et al*. Development of 3'3'-cyclic GMP-AMP enzyme linked immunoassay reveals phage infection reduces DncV activity. *Isr. J. Chem*. e202200084 (2023).

Appendix A: Supplemental Data

Compound	Cross Reactivity		
	3'3'-cGAMP ELISA	Cyclic di-GMP ELISA	Cyclic di-AMP ELISA
3'3'-cGAMP	100%	<0.01%	<0.01%
3'2'-cGAMP	0.018%	**	**
2'3'-cGAMP	0.006%	**	**
cyclic di-GMP	0.004%	100%	<0.01%
2'2'-cGAMP	0.002%	**	**
pApG	0.002%	<0.01%	<0.01%
ATP	<0.001%	**	**
GTP	<0.001%	<0.01%	**
AMP	<0.001%	**	**
GMP	<0.001%	**	**
cAMP	<0.001%	**	**
cGMP	<0.001%	<0.01%	**
cyclic di-AMP	<0.001%	<0.01%	100%
pGpG	**	0.10%	**
pG(2',5')pA	**	<0.01%	<0.01%
c[A(3',5')pA(3',5')pG(3',5')p]	**	<0.01%	0.015%
pApA	**	**	0.013%
c-hexa-AMP	**	**	<0.01%
c-ApUp	**	**	<0.01%
c-tetra-AMP	**	**	<0.01%

Table S1. Specificity of ELISAs to related compounds (obtained from assay manuals). **Not applicable

Explore these kits on Cayman's website for additional data, including:

- Complete data on kit performance:
  - Spike and recovery data
  - Linearity data
  - Cross reactivity tables
- Detailed assay kit protocols
- List of supplied reagents and materials