Cyclic-di-GMP assay kit: A first-in-class HTS assay for bacterial tolerance drug discovery

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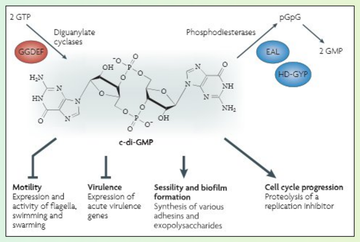
Introduction

- Biofilms play an important role in promoting bacterial tolerance and antibiotic resistance, two major threats in global public health.
- Despite NIH's estimate that 80% of all infectious diseases are caused by microorganisms in the biofilm state, there are no effective ways to prevent biofilm formation.
- Cyclic di-guanosine monophosphate (c-di-GMP) is a key second messenger involved in biofilm formation and enzymes that are involved in production and degradation of c-di-GMP are an attractive target for drug discovery.
- Current methods to monitor c-di-GMP, such as HPLC and MS, are slow and cumbersome, and requires organic extraction. Thus, there is a need for robust mix-and-use methods to accelerate the discovery of drugs that can inhibit biofilm formation and prevent bacterial tolerance.

Cyclic di-guanosine monophosphate (c-di-GMP)



Hengge, Nat. Rev. Microbiol., 2009

- A bacterial second messenger
- Key roles in bacterial motility, virulence, biofilm formation, cell cycle, antibiotic resistance, and tolerance
- c-di-GMP levels are regulated by diguanlyate cyclases (DGC) and phosphodiesterases (PDE)
- Biofilms are responsible for nosocomial, medical device linked infections and chronic infections in diseases such as cystic fibrosis, endocarditis, and chronic prostatitis
- c-di-GMP pathways are a potential targets for drug discovery

c-di-GMP analysis: current methods

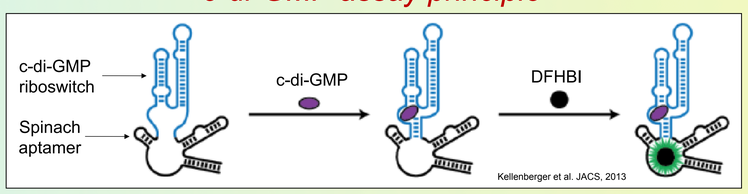
Methods:

- HPLC-MS
- FRET
- Fluorescence
- Circular dichroism
- Allosteric ribozymes
- Intercalator dyes

Drawbacks:

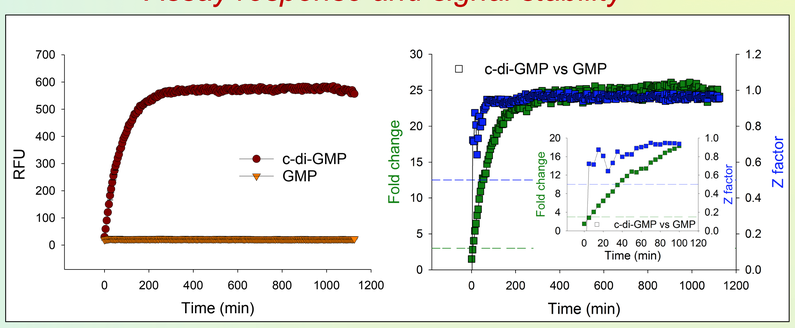
- Labor intensive and cumbersome
- Need of expensive equipment
- Require extensive optimization
- Poor detection range
- High background/low specificity
- Not easily adaptable for HTS

c-di-GMP assay principle



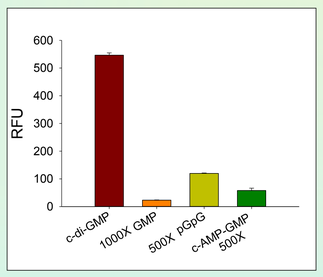
- Fluorogenic c-di-GMP assay based on Lucerna's Spinach™ technology
- Binding of c-di-GMP to the riboswitch module of the c-di-GMP sensor induces the folding of the Spinach aptamer and its binding to DFHBI
- DFHBI-bound c-di-GMP sensor emits green fluorescence detectable in the GFP/FITC channel and reflects the amount of c-di-GMP present

Assay response and signal stability



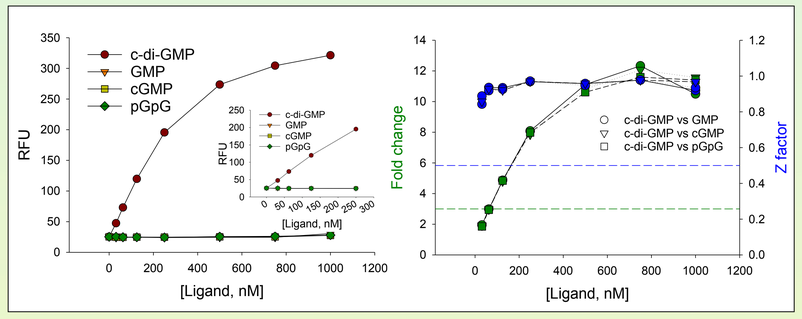
- Quick sensor response allows for fast c-di-GMP detection
- Stable fluorescent signal allows a large measurement window

Selectivity of c-di-GMP sensor



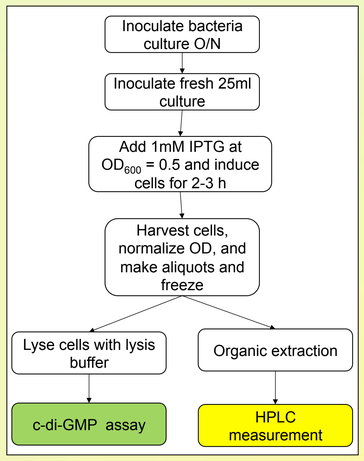
- Highly selective to c-di-GMP
- Assay meets HTS criteria with Z factor >0.9 and S/B >5, even in the presence of 500-1000 fold excess counter ligands (GMP, pGpG and c-AMP-GMP)

Sensitivity and selectivity of c-di-GMP

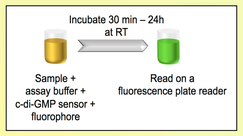


- Sensitive even at lower physiological concentrations
- Excellent dynamic range (50nM-1000nM)
- High selectivity for c-di-GMP vs. GMP, pGpG (c-di-GMP hydrolysis products)

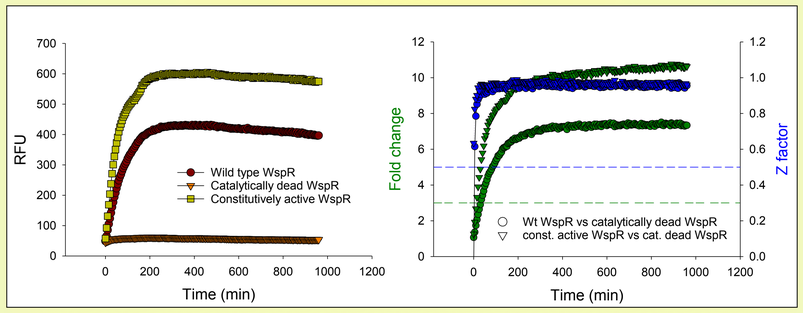
c-di-GMP analysis in bacterial lysates



- WspR, a diguanylate cyclase (DGC) from *pseudomonas aeruginosa,* uses two GTP molecules to produce c-di-GMP and pyrophosphate
- A bacterial cell model (E. *coli*) expressing wild type, catalytically dead, and constitutively active WspRs were used in the analysis
- c-di-GMP levels in bacteria was measured using the c-di-GMP assay
- c-di-GMP levels in bacteria were independently evaluated by HPLC and compared with the c-di-GMP assay estimates

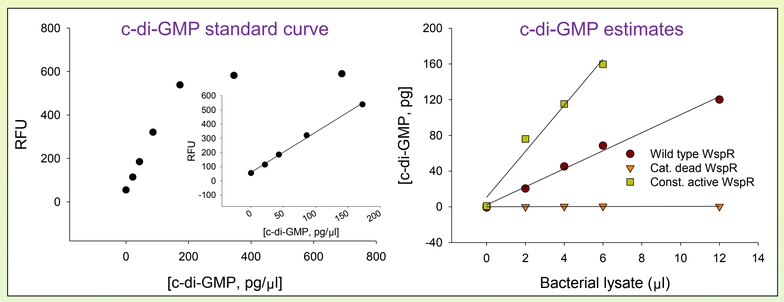


c-di-GMP assay performance in bacterial lysates



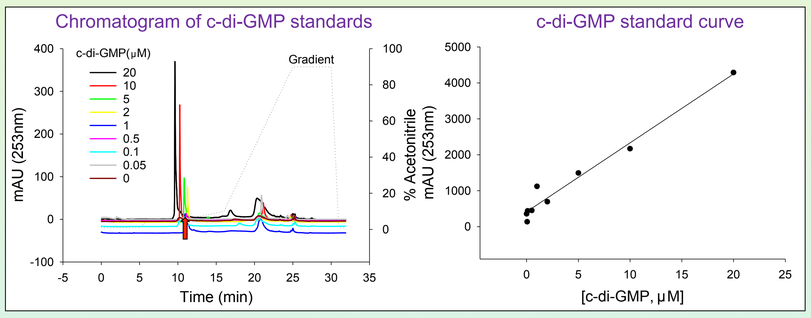
- Distinct signal response to differing levels of c-di-GMP in bacterial cells expressing wild type, catalytically dead and constitutively active WspR mutants
- Assay signal is stable over time, allowing a large window for measurement

Estimation in bacterial lysates using c-di-GMP assay



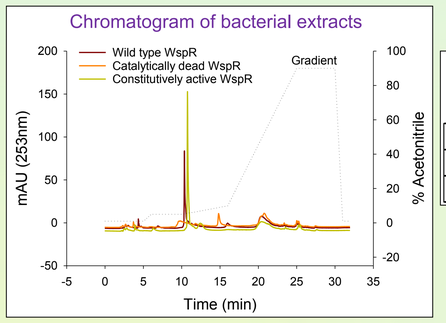
- Estimated c-di-GMP concentrations in bacteria expressing wild type, catalytically dead, and constitutively active WspR mutants

c-di-GMP standard curve using HPLC peak areas



- HPLC standard curve of c-di-GMP shows good fit with a r^2 >0.97

c-di-GMP estimation by c-di-GMP sensor vs HPLC

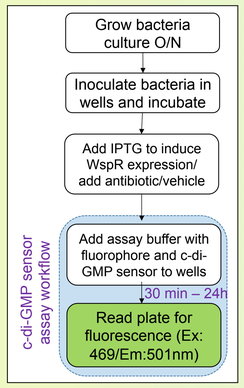


Comparison of c-di-GMP levels in bacterial lysate

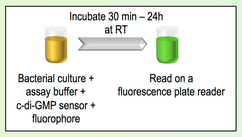
	c-di-GMP (pg/µl of lysate)	
WspR	HPLC	c-di-GMP sensor
Wild type	14.1	11.0
Const. active	32.2	27.6

- c-di-GMP levels estimated by Lucerna™ c-di-GMP assay and HPLC are comparable

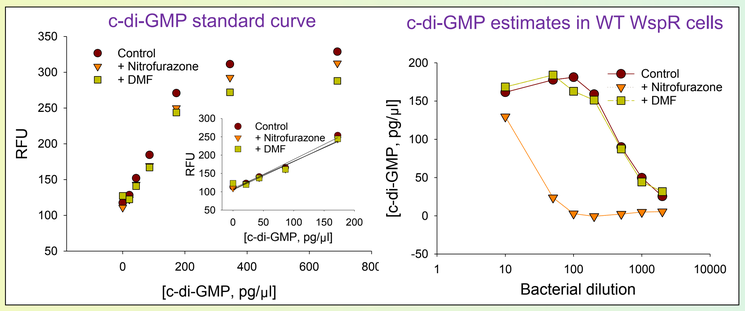
c-di-GMP analysis in bacteria cultures



- Bacteria expressing wild type WspR were plated at varying cell densities and treated with the antibiotic nitrofurazone, its vehicle dimethyl formamide (DMF), or left untreated
- c-di-GMP concentrations in bacteria were determined by the c-di-GMP assay in a homogenous format that requires no lysis or pelleting or wash steps

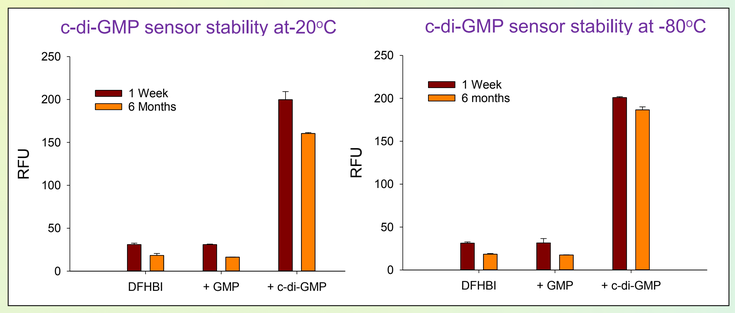


c-di-GMP assay in bacterial cultures



- c-di-GMP standards (0-1000nM) were assayed in LB media with antibiotic nitrofurazone (antibiotic), DMF (vehicle), or control (untreated)
- Presence of nitrofurazone, DMF, or LB media alone did not affect the performance of the c-di-GMP sensor assay
- c-di-GMP concentrations in bacteria were estimated based on the standard curve
- Changes in c-di-GMP levels by varying cell densities or by treatment with antibiotics are detected by the c-di-GMP sensor assay

c-di-GMP assay: storage stability



- c-di-GMP sensor is stable for 6 months at -20°C and -80°C

Summary

- Easy-to-use homogeneous assay format
- Quick signal response and prolonged signal stability
- Broad dynamic range (50nM - 1000nM)
- High selectivity towards c-di-GMP vs. counter ligands
- Compatible with biochemical and cell-based applications
- Assay is stable for at least 6 months when stored at -20 °C or lower
- Meets NCATS' HTS assay guidance criteria and suitable for drug discovery