CHAPTER FOUR

Chemiluminescent sensors for quantitation of the bacterial second messenger cyclic di-GMP

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Abstract

Chemiluminescent biosensors have been developed and broadly applied to mammalian cell systems for studying intracellular signaling networks. For bacteria, biosensors have largely relied on fluorescence-based systems for quantitating signaling molecules, but these designs can encounter issues in complex environments due to their reliance on external illumination. In order to circumvent these issues, we designed the first ratiometric chemiluminescent biosensors for studying a key bacterial second messenger,

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cyclic di-GMP. We have shown recently that these biosensors function both *in vitro* and *in vivo* for detecting changes in cyclic di-GMP levels. In this chapter, we present a practical and broadly applicable method for high-throughput quantitation of cyclic di-GMP in bacterial cell extracts using the high affinity biosensor tVYN-TmΔ that could serve as the "Bradford assay" equivalent for this bacterial signaling molecule.

1. Introduction

Second messenger molecules play key roles in regulating various aspects of bacterial lifestyle and behavior. Since bacteria depend on second messengers in order to adapt to changes in the environment, these signaling molecules have been characterized for their role in signal transduction. For instance, the nucleotide-based bacterial second messengers cAMP and (p)ppGpp have been heavily studied for their respective roles in catabolite repression and the starvation response in bacteria (Hengge, Gründling, Jenal, Ryan, & Yildiz, 2016). Another second messenger, cyclic di-GMP (*c*-di-GMP), is nearly ubiquitous in bacteria and serves as the master regulator of motility and biofilm formation, while also playing an important part in signal transduction pathways related to bacterial virulence and development (Romling, Galperin, & Gomelsky, 2013). Intracellular levels of *c*-di-GMP are regulated through the interplay of diguanylate cyclase (DGC) and phosphodiesterase (PDE) enzymes to tightly control the levels of *c*-di-GMP in a cell at any given time. In pathogenic bacteria, this tight control is vital for the regulation of virulence factors at various stages of infection in a mammalian host (Hall & Lee, 2018). In complex settings, such as in clinical isolates or *in vivo* systems, methods are needed to accurately quantitate *c*-di-GMP in a high-throughput manner.

One common method for studying intracellular levels of *c*-di-GMP relies on the use of liquid chromatography-mass spectrometry (LC/MS) analysis in bacterial cell extracts (Bahre & Kaever, 2017; Koestler & Waters, 2013; Spangler, Böhm, Jenal, Seifert, & Kaever, 2010). Although this analysis is highly sensitive, sample preparation and analysis times are long, limiting the throughput of this method. Mass spectrometry also is necessarily a bulk measurement method. Our lab and others instead have developed fluorescent biosensors that can dynamically measure *c*-di-GMP levels in live cells (Ho et al., 2013; Rybtke et al., 2017; Wang, Wilson, & Hammond, 2016). These fluorescent biosensors, using either RNA- or protein-based designs, have been used in applications such as the study of *c*-di-GMP dynamics in

response to zinc in *E. coli* using flow cytometry (Yeo, Dippel, Wang, & Hammond, 2018) and during cell cycle development in *Caulobacter crescentus* using fluorescence microscopy (Petersen, Mills, & Miller, 2019), respectively. However, due to their reliance on external illumination, fluorescent biosensors can encounter issues when used in contexts that lead to low light penetrance, high autofluorescence, or excessive photobleaching. Furthermore, for single cell and live cell imaging, these biosensors are genetically encoded, which precludes their use in analyzing genetically intractable organisms, mixed microbial communities, or clinical isolates.

To address some of these inherent challenges, we developed the first protein-based chemiluminescent biosensors for *c*-di-GMP. The first-generation design utilizes YcgR receptors for *c*-di-GMP fused to split-luciferase to generate intensity-based biosensors with nanomolar affinities for *c*-di-GMP (Dippel, Anderson, Evans, Deutsch, & Hammond, 2018; Saito et al., 2012). More recently, our second-generation design utilizes the same YcgR receptors fused between Venus fluorescent protein and NanoLuc (NLuc) luciferase to generate ratiometric biosensors (Dippel, Anderson, Park, Yildiz, & Hammond, 2020). Advantages of the second-generation biosensors include higher brightness, even greater affinity (picomolar) for *c*-di-GMP, and reliable ratiometric signal over time, up to an hour after substrate addition (Fig. 1). With these advanced chemiluminescent sensors, we have shown, for example, imaging of *c*-di-GMP activity in as few as ~1000 live *E. coli* cells in a phantom tissue model. These results highlight the growing potential for using chemiluminescent tools not only to track bacterial cells within tissues, but also to monitor the signaling status and activity of bacterial cells within host niches.

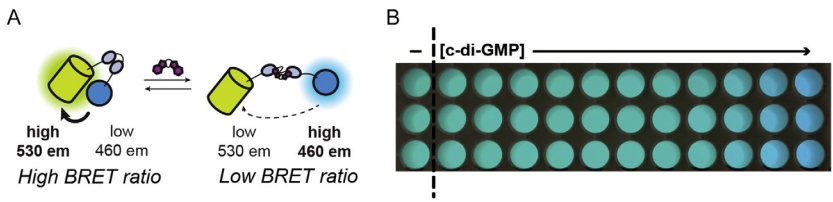

Fig. 1 Overview of BRET-based biosensors. (A) General schematic depicting the function of the tVYN-TmΔ biosensor for *in vitro* experiments. BRET efficiency decreases in the presence of cyclic di-GMP. (B) Cell phone picture taken of a 96-well plate containing biosensor in each well immediately after substrate addition. Biosensor incubated with only H_2O in the first column and with increasing concentrations of cyclic di-GMP in other columns. *Adapted with permission from* ACS Chemical Biology.

While tissue imaging remains a long-term goal, we have found in the meanwhile that our ratiometric biosensors also are highly useful for measuring *c*-di-GMP in bacterial cell extracts. This practical method requires no genetic manipulation and can provide rapid quantitation of *c*-di-GMP in genetically tractable and intractable organisms, mixed microbial communities, and clinical isolates. Due to its exceptional affinity, the tVYN-TmΔ biosensor can be applied to quantitate *c*-di-GMP with a lower limit of detection comparable to LC/MS but in a 96- or 384-well plate reader assay format. Due to its exceptional brightness, a medium-scale protein preparation (250 mL culture) typically yields sufficient biosensor reagent to analyze ~100,000+ samples in a 96-well format. Here we present the method for preparing the biosensor and quantitating *c*-di-GMP in *Vibrio cholerae* cell extracts using the tVYN-TmΔ ratiometric biosensor.

1.1 General equipment

Micropipette
Repeater pipette
Multichannel micropipette
Large refrigerated centrifuge (with rotors for 250 mL plastic bottles and 50 mL conical tubes for speeds up to 9200 rpm)
Millipore water filter for double-distilled H_2O (ddH_2O)
Heat block (42 °C)
1 L baffled flasks
UV/Vis spectrophotometer
Refrigerated shaking incubator
Nutator

1.2 General materials

Micropipette tips
1.5 and 2 mL microcentrifuge tubes
5 and 50 mL conical tubes
250 mL plastic bottles
Carbenicillin stock (Carb)
Kanamycin stock (Kan)
E. coli BL21 Star (DE3) cells
Miller's LB agar
Miller's LB broth
Petri dishes
Tryptone

Yeast extract
Sodium chloride (NaCl)
Potassium chloride (KCl)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
Tris base
Glycerol
Imidazole
Ethanol (EtOH)
Methanol (MeOH)
Glacial acetic acid
Liquid nitrogen
SDS
Bromophenol blue
Dithiothreitol (DTT)
Bovine serum albumin (BSA)

2. Biosensor expression and purification

To produce the biosensor for use in *c*-di-GMP quantitation assays, the tVYN-TmΔ biosensor is expressed in *E. coli* and purified using a Ni-NTA gravity flow column. Expressing the biosensor using the high copy $pRSET_B$ plasmid, which contains an N-terminal His_6 tag, gives good yields and reduces co-elution of truncated side products. Induction with IPTG, as opposed to auto-inducing media, enhances protein expression and yield (typical yields are ~25 μM biosensor per 250 mL culture). Due to its high affinity for *c*-di-GMP, which is present natively in *E. coli*, the biosensor is co-expressed with the phosphodiesterase PdeH to permit biosensor purification without *c*-di-GMP bound. A 250 mL expression culture typically yields sufficient biosensor for ~100,000+ well assays in a 96-well plate format using 3 nM biosensor per well.

2.1 Additional materials/equipment

$pRSET_B$-tVYN-TmΔ plasmid (Addgene ID: 149694)
pCOLA-PdeH plasmid (Addgene ID: 79165)
S.O.C. medium
1 M IPTG stock in ddH_2O
Lysozyme
PMSF
Sonic dismembrator
Ni-NTA resin

Chromatography columns with solid support
Amicon Ultra-15 centrifugal filter units (10 K MWCO)
Prestained protein ladder (~10–250 kDa)
Polyacrylamide gel electrophoresis equipment
Ammonium persulfate (APS)
Tetramethylethylenediamine (TEMED)
Coomassie Brilliant Blue G-250

Solutions that can be prepared in advance are listed below, while any solutions that should be freshly prepared are detailed in the procedure. ddH_2O should be used for any solutions containing water.

Carb/Kan LB agar plates

Component	Final concentration	Stock	Amount
Miller's LB agar	40 mg/mL	–	4 g
Carbenicillin	50 μg/mL	50 mg/mL	100 μL
Kanamycin	50 μg/mL	50 mg/mL	100 μL

Dissolve powder in 100 mL water with no antibiotics, autoclave, store at 4 °C. For pouring plates, microwave the solution until the agar is completely dissolved, add antibiotics after cooling, pour a thin layer into an empty Petri dish, and let cool to RT before plating. Any additional plates can be stored at 4 °C for later use.

Carb/Kan LB broth liquid medium

Component	Final concentration	Stock	Amount
Miller's LB broth	25 mg/mL	–	12.5 g
Carbenicillin	50 μg/mL	50 mg/mL	100 μL
Kanamycin	50 μg/mL	50 mg/mL	100 μL

Dissolve powder in 100 mL water with no antibiotics, autoclave, add antibiotics after cooling, store at RT.

Carb/Kan 2× YT medium (prepare in 250mL baffled flasks)

Component	Final concentration	Stock	Amount
Tryptone	16 mg/mL	–	4 g
Yeast extract	10 mg/mL	–	2.5 g
NaCl	5 mg/mL	–	1.25 g
Carbenicillin	50 μg/mL	50 mg/mL	100 μL
Kanamycin	50 μg/mL	50 mg/mL	100 μL

Add components except antibiotics, add water to 250 mL, autoclave, add antibiotics after cooling, store at RT. Set aside at least 2 mL for blanking the spectrophotometer before expression.

Biosensor lysis buffer

Component	Final concentration	Stock	Amount
Tris	50 mM	–	6.1 g
NaCl	150 mM	–	8.8 g
Imidazole	20 mM	–	1.4 g
Glycerol	5% (w/v)	50 mg/mL	63 g

Mix components in water, adjust pH to 7.5, add water to 1 L, sterile filter, store at 4 °C.

Biosensor wash buffer

Component	Final concentration	Stock	Amount
Tris	50 mM	–	6.1 g
NaCl	500 mM	–	29.2 g
Imidazole	20 mM	–	1.4 g
Glycerol	5% (w/v)	50 mg/mL	63 g

Mix components in water, adjust pH to 7.5, add water to 1 L, sterile filter, store at 4 °C.

Biosensor elution buffer

Component	Final concentration	Stock	Amount
Tris	50 mM	–	1.5 g
NaCl	150 mM	–	2.2 g
Imidazole	300 mM	–	5.1 g
Glycerol	5% (w/v)	50 mg/mL	15.8 g

Mix components in water, adjust pH to 7.5, add water to 250 mL, sterile filter, store at 4 °C.

Biosensor storage buffer

Component	Final concentration	Stock	Amount
HEPES	50 mM	–	11.9 g
KCl	100 mM	–	7.5 g
Glycerol	10% (w/v)	100 mg/mL	126 g

Mix components in water, adjust pH to 7.2, add water to 1 L, sterile filter, store at 4 °C.

2× SDS loading buffer

Component	Final concentration	Stock	Amount
Tris-HCl	100 mM	–	606 mg
SDS	4% (w/v)	–	2 g
Bromophenol blue	0.2% (w/v)	–	100 mg
Glycerol	20% glycerol (v/v)	–	10 mL
DTT	200 mM	1 M	10 mL

Add water to 50 mL, store at RT.

10× Tris-Glycine-SDS running buffer

Component	Final concentration	Stock	Amount
Tris	250 mM	–	30 g
Glycine	1.92 M	–	144 g
SDS	1% (w/v)	–	10 g

Add water to 1 L, store at RT, dilute to 1 × before running SDS-PAGE gel. The 1 × stock can be reused a few times for multiple gels before disposal.

Coomassie staining solution

Component	Final concentration	Stock	Amount
Coomassie Brilliant Blue	1 g/L	–	1 g
MeOH	50% (v/v)	–	500 mL
Glacial acetic acid	10% (v/v)	–	100 mL

Add water to 1 L, stir for 3–4 h, store at RT.

Coomassie destaining solution

Component	Final concentration	Stock	Amount
MeOH	50% (v/v)	–	500 mL
Glacial acetic acid	10% (v/v)	–	100 mL

Add water to 1 L, store at RT.

2.2 Procedures

Step 1: Plasmid transformation into competent bacteria (Estimated time: 18–22 h)

1.1 Add 10 ng each of pRSET$_B$-tVYN-TmΔ and pCOLA-PdeH to 15–20 μL of chemically competent *E. coli* BL21 Star (DE3) cells in a microcentrifuge tube thawed on ice.

1.2 Place the tube in a 42 °C heat block for 30 s to heat shock the cells. Remove the tube from heat and keep on ice for at least 10 s.

1.3 Add 250 μL S.O.C. medium to the tube and recover at 37 °C for at least 1 h with constant shaking at 250 rpm.

1.4 Plate 30 μL of cell suspension onto Carb/Kan LB agar plates and grow at 37 °C for 16–20 h.

Step 2: Biosensor expression (Estimated time: 2 days)

2.1 Inoculate overnight starter cultures by adding a single colony to 5 mL of Carb/Kan LB broth liquid medium in a standard culture tube. Incubate with shaking at 250 rpm, 37 °C for 16–20 h (see **Note 1**).

2.2 Use 2.5 μL of starter culture to inoculate 250 mL Carb/Kan 2× YT medium in a baffled flask. Grow each culture to an $OD_{600} \sim 1.0$ (check using excess 2× YT medium as a blank) with constant shaking at 250 rpm, 37 °C in an incubator (see **Note 2**). Transfer the flask to 4 °C for ~10 min to cool the culture and add 25 μL of 1 M IPTG to induce at a final concentration of 0.1 mM IPTG.

2.3 Transfer the flask to an incubator set to 18 °C and continue shaking at 250 rpm for 20 h to express the biosensor overnight.

Step 3: Cell lysis for biosensor purification (Estimated time: 90 min)

3.1 Harvest induced cells *via* centrifugation at 9200 rpm for 30 min. Dispose of the supernatant and resuspend cell pellets in 30 mL of biosensor lysis buffer in 50 mL conical tubes.

3.2 During the centrifugation step, prepare fresh stocks of 10 mg/mL of lysozyme dissolved in ddH_2O and 200 mM PMSF dissolved in EtOH. Add 900 μL of lysozyme and 150 μL of PMSF to the resuspended cells. Incubate on ice for at least 10 min.

3.3 Sonicate the cell suspension using a sonic dismembrator at 70% amplitude for 4 min (2 s on, 2 s off) to completely lyse the cells.

3.4 Transfer the cell lysate to a clean 50 mL conical tube and centrifuge for 40 min at 9200 rpm to pellet the cell debris.

Step 4: Ni-NTA resin column for biosensor purification (Estimated time: 90 min)

4.1 During the centrifuge step in **3.4**, add 4 mL of Ni-NTA resin into a new 50 mL conical tube.

4.2 Pour the crude protein lysate into the tube containing the Ni-NTA resin, taking care not to dislodge the cell debris pellet.

4.3 Let the supernatant mix with the resin at 4 °C for 1 h while rotating on a nutator. Periodically check the tube during the binding process to ensure the resin is homogenous with the added supernatant. If needed, shake the tube by hand to maintain homogeneity and put back on the nutator.

4.4 Set up a disposable chromatography column above a flask to collect flow-through at 4 °C. Pour the crude protein/resin mixture into the column, let the solution flow out by gravity, and dispose of the flow-through, or optionally save for future analysis with SDS-PAGE (see **Note 3**).

4.5 Wash the conical tube with 40 mL of biosensor wash buffer and pour the wash mixture with any residual resin into the column in 20 mL portions. Let the solution flow out by gravity and dispose of the wash flow-through, or optionally save for future analysis with SDS-PAGE. Make sure the resin bed covers the base of the column.

4.6 Add 8 mL of biosensor elution buffer to the column, taking care not to disturb the resin bed. Collect the elution fraction.

Step 5: Buffer exchange for biosensor storage (Estimated time: 2 h)

5.1 Transfer the elution fraction into a 15 mL Amicon Ultra-15 centrifugal filter unit (10 K MWCO) and ensure that the filter device is fitted into the collection tube before centrifugation. Balance the rotor with a dummy sample and centrifuge for 25 min at $5000 \times g$ to reduce the elution volume before buffer exchange (see **Note 4**). Dispose of the flow-through.

5.2 Add biosensor storage buffer into the unit up to the marked line on the tube ($\sim$10 mL). Centrifuge again for 25 min at $5000 \times g$ to start the buffer exchange.

5.3 Repeat step 5.2 until a total of 50 mL of storage buffer has been used for buffer exchange, disposing of the flow-through after each spin.

5.4 Pipette the remaining liquid slowly up and down to mix thoroughly. Transfer the liquid into a 2 mL microcentrifuge tube and dilute with storage buffer up to 2 mL final volume.

Step 6: Biosensor stock concentration determination (Estimated time: 20 min)

6.1 Using a nanovolume spectrophotometer, blank with storage buffer then measure the absorbance at 515 nm (Venus absorbance) of a 2.5 μL aliquot of the biosensor stock (see **Note 5**).

6.2 Calculate the concentration of the biosensor using Beer's Law and the extinction coefficient for Venus ($92{,}220\,M^{-1}\,cm^{-1}$). Typical concentrations following this protocol are in the range of 20–30 μM.

6.3 Save a small ~20 μL aliquot of the biosensor stock and store at 4 °C to run on the analysis gel. Make multiple aliquots of the remaining biosensor stock (for example, 20×100 μL aliquots of 20 μM), freeze them rapidly in liquid nitrogen, and store in a −80 °C freezer (see **Note 6**).

Step 7: Analysis of biosensor expression and purification using SDS-PAGE (Estimated time: 5 h)

7.1 Pour a 10% PAGE gel polymerized with 10% APS and TEMED between glass plates to a thickness of 0.75 mm. The Bio-Rad TGX FastCast system makes small analytical gels with 10 lanes.

7.2 Set up the electrophoresis equipment, place the gel in the chamber, and fill with 1 L of 1 × Tris-Glycine-SDS running buffer.

7.3 Prepare a 20 μL sample of 10 μM purified biosensor stock by diluting the stock aliquot with storage buffer. Add 20 μL of 2 × SDS loading buffer to this and other samples (see **Note 7**) and heat the tube(s) at 95 °C for 5 min to denature the proteins in the sample(s).

7.4 Load 10 μL of each SDS-protein sample into wells of the PAGE gel alongside a prestained protein ladder (~10–250 kDa). Run the gel at 140 V for ~45 min or until the lowest protein band of the ladder has migrated at least 80% of the distance of the gel (see **Note 8**).

7.5 Remove the gel from the chamber (see **Note 9**). Carefully separate the glass plates, peel off the gel, and place gel into a small tray. Rinse the gel with enough water to fully submerge it, dispose of the water, and repeat at least two more times until the gel is rinsed well.

7.6 Pour enough Coomassie staining solution into the tray to fully submerge the gel. Let the tray gently shake sideways on the nutator for at least 1 h at RT to fully stain the gel (see **Note 10**).

7.7 Pour out the Coomassie staining solution, taking care to keep the gel in the tray. Rinse out the excess staining solution with water a few times and dispose the liquid in a specially marked container for Coomassie waste. Submerge the gel in Coomassie destaining solution and gently shake sideways on a nutator for ~1–2 h at RT until bands are clearly visible (see **Note 11**).

7.8 Visually inspect the gel and confirm the expected biosensor protein was purified (expected MW ~73 kDa) by comparing to the prestained protein ladder (see **Note 12**).

2.3 Notes

1. The starter culture can be grown to confluence and used to inoculate the 2× YT expression medium sooner than 16–20 h, the timing in this case is for the convenience of the induction step, see **Note 2**.
2. The culture can take from 2 to 6 h to reach the require $OD_{600} \sim 1.0$ for induction. It is advised to inoculate early in the day in case the growth takes longer than expected.
3. The initial flow-through and subsequent washes can be pushed through the column to save time. This is done by taking an electronic pipettor attached to a serological pipette and forming a seal with the column to forcefully push air through the column. However, ensure when doing this that the buffer does not go below the resin bed.
4. The volume after centrifugation should be ~2–3 mL. This volume should remain consistent after repeated storage buffer additions and centrifugations.
5. Due to the glycerol content of the storage buffer, care needs to be taken when pipetting small volumes and ensure that all liquid is being expelled from the pipette before absorbance measurements.
6. Aliquots are recommended to prevent degradation of biosensor performance from multiple freeze-thaw cycles. After thawing, these aliquots can be stored at 4 °C for a couple of days for use before disposal.
7. Multiple different fractions from purification (flow-through, washes, elution) can be collected and run on the PAGE gel alongside the final concentrated solution. The volumes used to dilute the proteins after adding 2× SDS loading buffer can be altered as long as 10 μL of 5 μM protein is loaded into the gel.

8. The buffer level while running the gel must be carefully monitored to ensure the running buffer in between the gel and the buffer dam does not leak and fall below the sample wells in the gel. If the buffer does fall below this line, the run can be stopped, buffer can be replaced, and the run can resume without any problems.
9. The running buffer and Coomassie staining solution can be poured into their original containers for reuse later.
10. Staining can be sped up by covering the gel tray with plastic wrap and microwaving it for ~15 s before rotating on the nutator.
11. Destaining can be sped up by tying a couple of Kimwipes into a knot and placing them in the tray with the gel. Change out the Kimwipes every 30 min to help the process.
12. If a sharp band is visible at ~30 kDa then that likely indicates the presence of Venus protein as a degradation product. Because the concentration of biosensor was estimated using Venus absorbance, the presence of Venus protein must be compensated for in order to estimate the "active" concentration of full-length biosensor for experimental use.

3. Generating a standard curve for quantitating *c*-di-GMP

Before running extract samples, a standard curve for the biosensor needs to be generated in order to quantitate *c*-di-GMP. Although the biosensor shows consistent performance *in vitro* in our hands, a new standard curve should be generated for each new biosensor protein preparation to verify its activity prior to measuring any samples (Fig. 2). Depending on reagent availability, the following calculations for making stock solutions and combining can be adjusted, but each well must have 100 μL total volume with all reagents at the given concentration before substrate addition. Reagents that must be freshly prepared are described in Step 1.

3.1 Additional materials/equipment

Luminescent plate reader with temperature control
Cyclic di-GMP
h-coelenterazine luminescent substrate (h-CTZ)
Sodium ascorbate
96-well LUMITRAC 600 plates

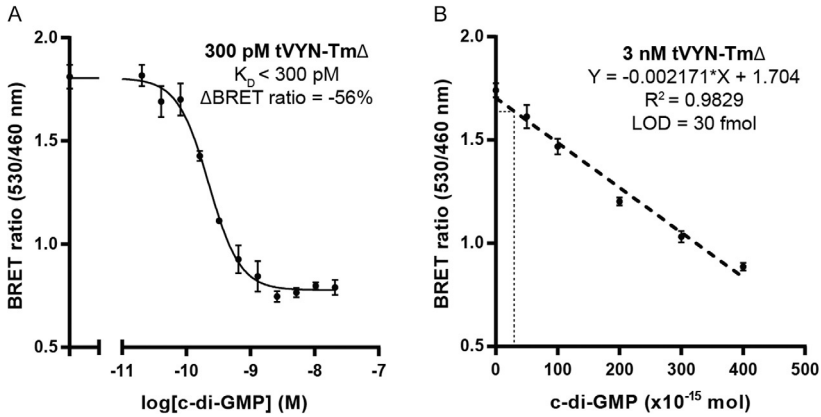

Fig. 2 Characteristics of the tVYN-TmΔ biosensor. (A) C-di-GMP affinity measurements with 300 pM tVYN-TmΔ. Measured K_D is less than 300 pM. (B) Example standard curve generated using the described procedure with 3 nM tVYN-TmΔ. *Adapted with permission from* ACS Chemical Biology.

Solutions that can be prepared in advance are listed below, while any solutions that should be freshly prepared are detailed in the procedure. ddH_2O should be used for any solutions containing water.

h-CTZ stock solution

Component	Final concentration	Stock	Amount
h-coelenterazine	6.15 mM	–	5 mg
Cold EtOH	–	–	2 mL

Mix well until fully dissolved, aliquot into ~10 microcentrifuge tubes, store at −80 °C.

5× biosensor assay buffer

Component	Final concentration	Stock	Amount
HEPES	250 mM	–	14.9 g
KCl	500 mM	–	9.3 g

Mix components in water, adjust pH to 7.2, add water to 250 mL, sterile filter, store at RT.

3.2 Procedures

Step 1: Preparation of fresh assay components (Estimated time: 30 min)

1.1 Dissolve 198 mg of sodium ascorbate powder in 1 mL ddH_2O to prepare fresh 1 M sodium ascorbate solution.

1.2 To prepare 1 mL of fresh h-CTZ substrate at 60 μM (6 ×) in assay buffer (h-CTZ assay solution), combine the following in a 1.5 mL microcentrifuge tube (see **Note 1**). This solution must be left at RT for at least 30 min before use to allow for equilibration:

200 μL 5 × biosensor assay buffer
300 μL 1 M sodium ascorbate
9.75 μL 6.15 mM h-CTZ stock solution
490.25 μL ddH_2O

1.3 Dissolve 15.4 mg DTT in 1 mL ddH_2O to prepare fresh 100 mM DTT (10 × DTT).

1.4 Dissolve 10 mg BSA in 1 mL ddH_2O to prepare fresh 1% BSA (10 × BSA).

1.5 Thaw an aliquot of biosensor stock to prepare fresh biosensor assay solution, which contains additives that improve biosensor signal stability. Combine the following in a 5 mL conical tube:

875 μL 5 × biosensor assay buffer
437.5 μL 10 × DTT
437.5 μL 10 × BSA
1–10 μL biosensor (to 3.75 nM final concentration, see **Note 2**)
ddH_2O to 3.5 mL (add last)

Step 2: Ligand and plate preparation (Estimated time: 30 min)

2.1 Prepare 150 μL each of standard concentration stocks of *c*-di-GMP at 2.5, 5, 10, 15, and 20 nM by serial dilution.

2.2 In each sample well on the LUMITRAC plate, combine 20 μL of each *c*-di-GMP standard or ddH_2O as negative control with 80 μL of fresh biosensor assay solution. We suggest testing six replicates for each sample (see **Note 3**). This standard curve consists of 0, 50, 100, 200, 300, and 400 fmol of *c*-di-GMP.

2.3 Incubate the plate at 28 °C for at least 10 min to allow for binding equilibration before measuring luminescence.

Step 3: BRET ratio measurements in plate reader (Estimated time: 10 min)

3.1 Set up the plate reader with the following settings (see **Note 4**):
Time: 5 min total, read in 30 s intervals
Emission wavelengths: 460, 530 nm
Shake: 3 s before each read
Temperature: 28 °C

3.2 Using a repeater pipette, manually add 20 μL of 6× h-CTZ assay solution to each well in quick succession. Start the full plate read. (see **Notes 5 and 6**).

Step 4: Standard curve analysis (Estimated time: 1 h)

4.1 Export the raw data as a CSV file to begin data analysis in a standard spreadsheet software. Organize the data such that 530 nm/460 nm BRET ratios are calculated using luminescence intensities at each wavelength for each time point at each *c*-di-GMP concentration.

4.2 Plot the data to visualize BRET ratio trends over the course of the 5 min kinetic read. For standard curve generation, a time point should be chosen whereby the ratio reaches a stable value with little associated error. The biosensor typically needs time to equilibrate and reach a stable ratio after the addition of substrate, as usually evidenced by the high error associated with measurements at early time points. For these experiments, using the BRET ratio at the 5 min mark at the end of the read usually suffices for creating the standard curve.

4.3 Plot the BRET ratio at the chosen time point for each concentration of *c*-di-GMP, verifying that the ratio decreases as the *c*-di-GMP concentration increases. Run a linear regression analysis to generate the standard curve with a linear equation, where X represents the amount of *c*-di-GMP (in fmol) and Y represents the BRET ratio. Verify that the R^2 value indicates a good linear fit before moving on to test extract samples (see **Note 7**).

3.3 Notes

1. We get sufficient signal when using h-coelenterazine for this assay, but furimazine is the native substrate for NanoLuc.
2. Depending on the stock concentration, it may be necessary to dilute the stock 1:10 to have a good working concentration. The amount of fresh biosensor assay solution prepared here (3.5 mL) is sufficient for 43 sample wells.
3. Although we describe using six technical replicates of each concentration for generating the standard curve, a minimum of three replicates can be used to save on materials.

4. We run these assays for 5 min to allow enough time for any error to be resolved in the earlier time points. Exact settings we use with a Molecular Devices SpectraMax i3x plate reader:
   Kinetic read—Time 5:00, Interval: 00:31, Reads: 10
   Luminescence—Lm1: 460 nm, Lm2: 530 nm
   Plate type—96 Well Greiner white-optimized
   Shake—medium, before first read for 3 s
   PMT and Optics—Integration Time: 200 ms, Read Height: 0.5 mm
   Temperature: 28 °C
5. A repeater pipette is highly recommended during this step to keep the time between substrate addition in the first and last wells under 30 s.
6. As an optional way to verify that the wells have a sufficient amount of biosensor, turn off the lights after adding h-CTZ into the plate to see the brightness. Wells containing different amounts of *c*-di-GMP should be slightly different colors due to the changing BRET ratios and intensity of NanoLuc relative to Venus (Fig. 1).
7. Following this protocol, the standard curve has consistently been shown to produce linear fits with R^2 values >0.95.

4. Bacterial cell extract analysis

We have used a ratiometric BRET biosensor to quantify *c*-di-GMP concentrations in *Vibrio cholerae* extracts from two conditions that have been demonstrated using LC-MS/MS to have differential intracellular concentrations of *c*-di-GMP. In one case, extracts were generated from wild type *V. cholerae* cells grown with and without 0.4% bovine bile, as it was previously shown that bile increases *c*-di-GMP concentrations (Koestler & Waters, 2014). In a second case, quorum sensing (QS) control of *c*-di-GMP in *V. cholerae* was explored. QS is a density-dependent bacterial cell-cell communication mechanism that allows bacteria to alter global gene expression as cultures transition from low-cell density to high-cell density. C-di-GMP levels are higher in the low-cell density state relative to high-cell density, which drives *V. cholerae* biofilm formation (Waters, Lu, Rabinowitz, & Bassler, 2008). Thus, extracts were generated from wild type *V. cholerae* or an isogenic Δ*hapR* mutant, which locks the cells into the low-cell density state.

The data shown were obtained using an early version ratiometric BRET biosensor, VYN-Nt (Fig. 3). Given its improved affinity and signal change, we recommend using the tVYN-TmΔ biosensor, which has been applied recently to *c*-di-GMP quantitation in *V. cholerae* extracts under

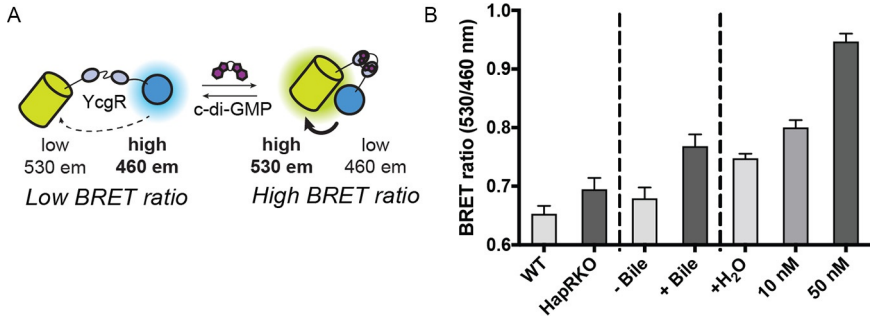

Fig. 3 BRET measurements of *V. cholerae* extract samples using the VYN-Nt biosensor. (A) VYN-Nt behaves as a positive change biosensor and produces high BRET ratios under high cyclic di-GMP conditions. (B) BRET ratios measured for extracts produced from *V. cholerae* grown with high or low-cell density (WT *vs* HapRKO) and WT grown with or without bile, compared to cyclic di-GMP standards as controls. *Adapted with permission from* ACS Chemical Biology.

additional growth conditions (Dippel et al., 2020). Since different growth conditions may affect cell densities, we also provide an optional method to normalize the *c*-di-GMP measurements to total protein content.

4.1 Additional materials/equipment

Acetonitrile
Methanol
Formic acid
Glass test tubes (2 and 8 mL)
Savant DNA120 SpeedVac® Concentrator
Optional: BCA protein assay kit
All assay components from part 3.1 are required for this procedure.

4.2 Procedures

Step 1: Cell extraction (Estimated time: 24 h)

Extraction buffer

Component	Final concentration	Stock	Amount
Acetonitrile	40% (v/v)	–	40 mL
Methanol	40% (v/v)	–	2 mL
Formic acid	4.2% (v/v)	–	4.2 mL

Add water to 100 mL in a glass bottle, store at −20 °C.

1.1 Determine how many extract conditions will be tested. For accurate results, we recommend preparing at least three biological replicates for each condition.

1.2 Grow bacteria using standard BSL-2 safety precautions in 3 mL of LB media with or without 0.4% bile in an 8 mL glass test tube (18 × 150 mm) at 35 °C or an appropriate temperature with agitation at 210 revolution per minute (RPM).

1.3 Once cells reach a desired OD_{600} remove a 1.5 mL sample and transfer to a 2.0 mL tube. For example, at an OD_{600} of ~0.3 an exponential phase sample could be collected and at an OD_{600} of ~2.0 a stationary phase sample could be collected to compare *c*-di-GMP levels between these two growth phases. Optionally, use the rest of the culture volume to run a BCA protein assay for later normalization of *c*-di-GMP concentration to total protein content.

1.4 Pellet cells by spinning down the sample in a 2.0 mL test tube at 13,000 × *g* in a table-top microcentrifuge for 3 min.

1.5 Remove the supernatant by decanting and gently tapping the 2.0 mL test tube upside down against an absorbent towel.

1.6 Add 100 μL of cold extraction buffer to each sample and vortex briefly until the solution is homogenous. Incubate suspension at −20 °C for 20 min.

1.7 Pellet cells by spinning down the sample in a 2.0 mL test tube at 13,000 × *g* in a table-top microcentrifuge for 5 min and then transfer supernatant to a 0.2 mL PCR test tube.

1.8 Evaporate extraction buffer, concentrate and dry the sample overnight by using a refrigerated Savant DNA120 SpeedVac® Concentrator.

Step 2: Cell extract dilutions (Estimated time: 40 min)

2.1 Resuspend cell extracts in 100 μL ddH_2O.

2.2 Multiple dilutions of each extract must be made in order to ensure that the extracts contain levels of *c*-di-GMP within the linear range of the biosensor. We typically make the following serial dilutions in ddH_2O: 1:5, 1:10, 1:20, 1:40, 1:80 (see **Note 1**). At least 50 μL of extract at each dilution must be prepared to test two technical replicates for each biological replicate.

Step 3: Preparation of substrate and biosensor solutions (Estimated time: 30 min)

3.1 Prepare fresh coelenterazine-h substrate solution as described in Section 3.2, **Step 1.2**. The protocol will prepare 1 mL of substrate, but this value can be adjusted depending on the number of samples

and replicates being tested. Each well in the 96-well plate requires 20 μL of substrate (see **Note 2**).

3.2 Prepare fresh biosensor stock solution as described in Section 3.2, **Step 1.5**. The protocol will prepare 3.5 mL of biosensor solution, but this value can be adjusted depending on the number of samples and replicates being tested. Each well used in the final 96-well plate requires 80 μL of biosensor solution (see **Note 2**).

Step 4: Plate preparation (Estimated time: 30 min)

4.1 In each well of the plate that is being used, follow Section 3.2, **Step 2.2** and combine 80 μL of biosensor solution with 20 μL of a diluted extract sample.

4.2 Incubate the plate at 28 °C for at least 10 min to equilibrate.

4.3 Set up the plate reader according to the same parameters as in Section 3.2, **Step 3.1** and start luminescence measurements after adding 20 μL of h-CTZ solution to each well.

Step 5: Quantitation of c-di-GMP concentrations (Estimated time: 20 min)

5.1 After collecting BRET ratios at each time point for each dilution, use the same time point that was chosen in Section 3.2, **Step 4.2** to calculate the average BRET ratio for each diluted extract sample (see **Note 3**).

5.2 For each strain or condition being tested, choose a dilution that gives an average BRET ratio within the linear range of the biosensor, which is ~0.9–1.6 (see **Note 4**). Input the BRET ratio into the standard curve equation to determine the amount of *c*-di-GMP (in fmol) present in the 20 μL of diluted extract.

5.3 Back-calculate the concentration of *c*-di-GMP (in nM) present in the undiluted extract by dividing the amount of *c*-di-GMP determined in **Step 5.2** by 20 μL and multiplying by the dilution factor for that sample.

5.4 Divide the concentration by 1000 to convert from nanomolar to pmol/μL.

5.5 To determine the pmol *c*-di-GMP/mL culture, multiply the value determined in **Step 5.4** by the resuspended extract volume (100 μL) and divide by the cell culture volume (1.5 mL).

5.6 Optionally, normalize the total pmol *c*-di-GMP/mL culture concentration to total protein content by using the results of the BCA assay to determine the pmol *c*-di-GMP/mg protein concentration value for each extract condition.

4.3 Notes

1. If the measured BRET ratio does not appear in the linear range of the biosensor of ~0.9–1.6 (verified by the standard curve) then additional dilutions will need to be tested.
2. Prepare at least 10% more than the required amount of each solution to test every extract condition without running out of h-CTZ and/or biosensor.
3. Since the BRET ratio for this biosensor is extremely stable ~30 s after substrate addition, any time point after 30 s can likely be chosen for analysis, but it is best to choose the same time point used for standard curve generation to maintain consistency.
4. In general, choosing BRET ratios at a more concentrated condition is better to reduce possible pipetting error that can result from preparing more dilute samples.

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