CHAPTER EIGHT

In Vitro Analysis of Riboswitch–Spinach Aptamer Fusions as Metabolite-Sensing Fluorescent Biosensors

Colleen A. Kellenberger*, Ming C. Hammond*,†,1
*Department of Chemistry, University of California, Berkeley, California, USA
†Department of Molecular & Cell Biology, University of California, Berkeley, California, USA
[1]Corresponding author: e-mail address: mingch@berkeley.edu

Contents

Abstract

The development of fluorescent biosensors has been motivated by the interest to monitor and measure the levels of specific metabolites in live cells in real time. Common approaches include fusing a protein-based receptor to fluorescent proteins or synthesizing a small molecule reactive probe. Natural metabolite-sensing riboswitches also have been used in reporter-based systems that take advantage of ligand-dependent regulation of downstream gene expression. More recently, it has been shown that RNA-based fluorescent biosensors can be generated by fusing a riboswitch aptamer to the *in vitro* selected Spinach aptamer, which binds a cell-permeable and conditionally fluorescent molecule. Here, we describe methods to design, prepare, and analyze riboswitch–Spinach aptamer fusion RNAs for ligand-dependent activation of fluorescence *in vitro*. Examples of procedures to measure fluorescence activation, ligand

Methods in Enzymology, Volume 550
ISSN 0076-6879
http://dx.doi.org/10.1016/bs.mie.2014.10.045

binding selectivity and affinity, and binding kinetics are given for a cyclic di-GMP-responsive biosensor. The relative ease of *in vitro* RNA synthesis and purification should make this method accessible to other researchers interested in developing riboswitch-based fluorescent biosensors.

1. INTRODUCTION

Many soluble signaling molecules dynamically regulate cellular physiology in response to changes in environmental conditions. Thus, observing correlations between the concentration of a specific signaling molecule and input stimuli or output responses is an important step toward deconvoluting often complex signal transduction pathways. Furthermore, there is growing appreciation that small molecule signals function in a spatially and temporally resolved manner inside cells (Newman, Fosbrink, & Zhang, 2011). The development of tools to tackle outstanding questions in the cell biology and *in vivo* biochemistry of small molecule signals or metabolites is an exciting and rapidly advancing research area.

An ideal method for tracking the dynamics of a small molecule in cells is through development of a biosensor, which is a receptor that specifically recognizes a small molecule ligand and provides a dynamic and measurable output in response to changing concentrations of the ligand. Most common biosensors are protein-based fluorescent turn-on or FRET sensors (Newman et al., 2011). While proteins are advantageous because they are genetically encodable and naturally specific for the metabolite of interest, it is often difficult to find a suitable receptor and to engineer a fusion construct that shows a large ligand-dependent change in signal. Another powerful approach toward detecting small molecule flux is the development of small molecule reactive probes (Liu, He, & Guo, 2013). However, small molecule probes with specific chemical reactivity toward a target analyte must be chemically synthesized and must be able to permeate through the cell membrane (Domaille, Zeng, & Chang, 2010; Liu et al., 2013). They are often designed to react irreversibly with the target analyte, although this is not always the case.

As an alternative, riboswitches naturally possess many of the traits required of biosensors. These RNAs are naturally evolved to recognize a specific metabolite and upon ligand binding, undergo a conformational change (Serganov & Nudler, 2013). Riboswitches have previously been used as small molecule reporters by placing a reporter gene, such as GFP

or luciferase, under the control of the riboswitch (Fowler, Brown, & Li, 2010). While these reporters are metabolite specific and genetically encodable, they lack the dynamic response of a biosensor, as the lifetime of the protein reporter does not correspond to the concentration of the metabolite.

Recently, Jaffrey and coworkers reported the development of the Spinach aptamer (Paige, Wu, & Jaffrey, 2011) that was evolved to bind the cell-permeable molecule 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) and to elicit fluorescence. The crystal structure of Spinach bound to DFHBI has recently been solved and has resulted in a revision of the secondary structure model (Fig. 1; Deigan Warner et al., 2014; Huang et al., 2014). Spinach can be engineered to fluoresce in response to metabolite binding through replacement of the second stem-loop of Spinach with other RNA aptamers (Kellenberger, Wilson, Sales-Lee, & Hammond, 2013; Nakayama, Luo, Zhou, Dayie, & Sintim, 2012; Paige, Nguyen-Duc,

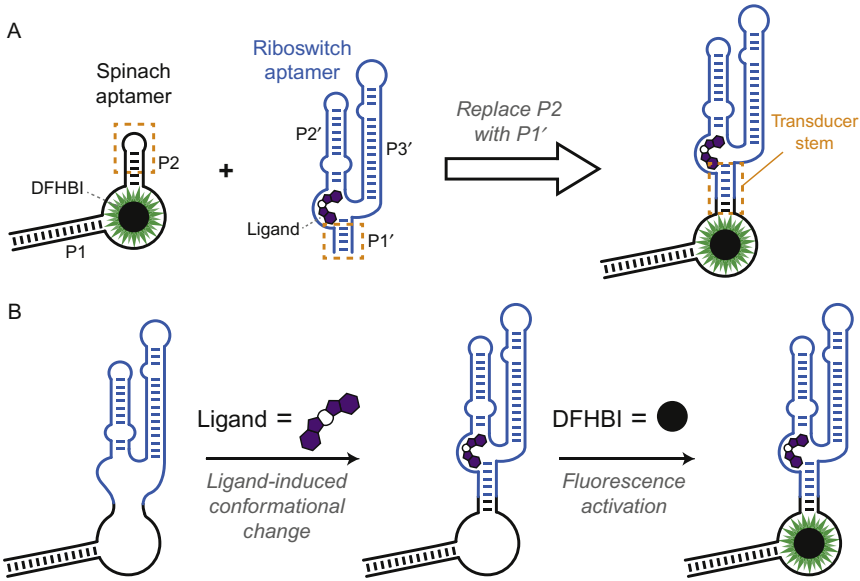

Figure 1 Design of Spinach-based biosensor and model of fluorescence activation. (A) The riboswitch aptamer is inserted into the Spinach scaffold in place of the P3 stem of Spinach. The P1′ stem of the Vc2 riboswitch aptamer, which is stabilized upon binding of ligand, serves as the transducer stem between the riboswitch and Spinach aptamers. (B) Binding of ligand to the riboswitch aptamer induces a conformational change in the transducer stem that enables the Spinach aptamer to bind DFHBI and elicit fluorescence.

Song, & Jaffrey, 2012). Binding of Spinach to DFHBI is dynamic and dependent on the ligand-bound state of the riboswitch aptamer, thus providing a direct fluorescent read-out of fluctuating metabolite concentrations. Furthermore, in comparison to protein-based fluorescent biosensors, it is more tractable to use RNA-based biosensors as both *in vivo* and *in vitro* sensors of metabolites. While use of a protein biosensor *in vitro* requires extensive purification of the protein from cells and any bound metabolites, RNA-based biosensors can be readily synthesized through *in vitro* transcription and directly used for analyses.

Following this strategy, here we describe the development of an RNA-based biosensor for the bacterial signaling molecule, cyclic di-GMP (c-di-GMP), by fusing Spinach to the Vc2 aptamer, a GEMM-I class riboswitch (Kellenberger et al., 2013). Binding of c-di-GMP to Vc2 stabilizes the transducer stem to Spinach, thus enabling Spinach to bind DFHBI and elicit fluorescence (Fig. 1). The development of this biosensor provides a sensitive method for studying the regulation of c-di-GMP, which is an important bacterial second messenger involved in the regulation of bacterial biofilms and pathogenesis (Römling et al., 2013).

1.1. General equipment

Micropipettor
Multichannel micropipettor
Vortex mixer
Microcentrifuge with temperature control
Millipore water filter with a BioPak unit

1.2. General materials

Micropipettor tips
Filter-tip micropipettor tips
1.5 mL microcentrifuge tubes
Sterile filter units
dNTPs: dATP, dCTP, dGTP, dTTP
rNTPs: ATP, CTP, GTP, UTP
Magnesium chloride ($MgCl_2$)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
Sucrose
Bromophenol blue
Xylene cyanol

SDS
Tris base
Boric acid
Sodium chloride (NaCl)
Potassium chloride (KCl)
EDTA, pH 8.0
Sodium carbonate (Na_2CO_3)
Acetic acid
T7 RNA polymerase
Inorganic pyrophosphatase
Ethanol (EtOH)
Sodium hydroxide (NaOH)
Acetic Acid
3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI)
Dimethyl sulfoxide (DMSO)
Cyclic di-GMP
National Diagnostics Sequagel mix
GE healthcare G-25 spin purification column
Dithiothreitol (DTT)
Glycerol
Double-distilled H_2O (ddH_2O, from Millipore water filtration system, 18.2 $M\Omega$ at 25 °C)

2. DESIGN AND PREPARATION OF AN RNA-BASED FLUORESCENT BIOSENSOR

For the fluorescent biosensor to function, ligand binding to the riboswitch aptamer, which is the domain that specifically recognizes a small molecule, must lead to a change in fluorescence, preferably a turn-on of fluorescence. This is affected by fusing the riboswitch aptamer to the Spinach aptamer in such a way that ligand binding enhances the affinity for DFHBI (Fig. 1). Formation of the Spinach P2 stem is required for binding of DFHBI, so connecting a riboswitch aptamer at this location creates a "transducer" stem that is affected by ligand binding (Paige et al., 2012). While others have screened artificial transducer stems and found some functional sequences (Nakayama et al., 2012; Paige et al., 2012), we recommend using the natural P1 stem of a riboswitch aptamer, in this case Vc2 (labeled as P1′ in Fig. 1), as a starting point for biosensor design. There are two main reasons, the first being that many riboswitches utilize conformational

changes involving the P1 stem to modulate gene expression (Blount, Wang, Lim, Sudarsan, & Breaker, 2007; Mandal & Breaker, 2004), so the natural sequence has evolved to perform the function of a transducer. Secondly, the natural P1 stem has evolved as part of the riboswitch fold, whereas artificial stems may perturb the folding pathway or stability of the RNA structure. For example, Vc2-Spinach biosensors incorporating artificial P1 stems showed drastic loss of ligand binding affinity (Nakayama et al., 2012).

In the case that one selects the natural P1 stem to serve as the transducer, there are still some design choices as to optimal length, inclusion or deletion of non-base-paired nucleotides, and mutations to increase or decrease stem stability. We typically screen several different construct designs varying these types of parameters to identify a working biosensor sequence. In our experience, even relatively modest changes such as comparing 4-nt versus 5-nt length transducer stems can have a large impact on the extent of ligand-induced fluorescence activation. These effects from modifying the transducer stem are not only due to altering the thermodynamic stability of the stem, because the three-dimensional arrangement of the two aptamers are likely to be important as well. An A-type RNA double helix has an average rotation of 37.4° per base-pair (Cruse et al., 1994), so a difference of one base-pair changes the relative orientation of the two aptamers and may lead to misfolding due to steric clash between folded domains. A long transducer stem would increase separation between the two aptamers but may be overstabilized, leading to higher background fluorescence.

The process of transducer stem optimization is similar to the need to screen different linker regions in the construction of FRET-based protein biosensors (Zhou, Herbst-Robinson, & Zhang, 2012). One major benefit is that *in vitro* transcription of RNA-based biosensors from a DNA template, followed by PAGE gel purification, is a straightforward procedure. Furthermore, as riboswitch aptamer sequences are usually 50–200 nt in length and the Spinach aptamer is 73 nt in length, full-length DNA templates of the riboswitch–Spinach aptamer fusions can be purchased as single-stranded oligonucleotides from commercial vendors or assembled by the overlapping PCR method (Nelson & Fitch, 2011).

2.1. Preparation of DNA templates of riboswitch–Spinach aptamer fusion

Shown below is the sequence of the WT Vc2-Spinach biosensor construct. The capitalized nucleotides indicate the 5′ and 3′ portions of the Spinach aptamer (black in Fig. 1) and the lowercase nucleotides represent the Vc2 riboswitch (blue (gray in the print version) in Fig. 1). The transducer stem,

consisting of the top four base-pairs of the natural Vc2 P1′ stem and containing a single nucleotide bulge, is underlined. Bolded regions anneal to the universal forward and reverse primers used in PCR that generates and amplifies a double-stranded template (from a single-stranded commercial oligonucleotide) and introduces the T7 promoter sequence. To design another biosensor, insert the desired aptamer sequence in place of the lowercase WT Vc2 sequence. This procedure works best for aptamers with a pairing stem that comprises the 5′ and 3′ ends of the aptamer.

WT Vc2-Spinach sequence: 5′-**GACGCGACTGAATGAAATGG** TGAAGGACGGGTCCAcacgcacagggcaaaccattcgaaagagtgggacgcaaagcctc cggcctaaaccagaagacatggtaggtagcggggttaccgatgTTGTTGAGTAGAGTGT **GAGCTCCGTAACTAGTCGCGTC**-3′

Universal forward primer (underlined is minimal T7 promoter with additional bases upstream to enhance polymerase binding and initiation in lower caps): 5′-ccaagTAATACGACTCACTATAGACGCGACTGAAT GAAATGG

Universal reverse primer: 5′ GACGCGACTAGTTACGGAGCTC

Generating the construct through overlapping extension PCR from shorter oligonucleotides is particularly useful if one wants to test a series of mutations or changes to the same biosensor, or if the biosensor constructs are greater than 200 nt. In the procedure described below, universal primers that anneal to the flanking Spinach sequences are used to generate a double-stranded template and introduce the T7 promoter sequence.

Notes and tips:

- T7 RNA polymerase prefers to initiate transcription at G nucleotides (Kuzmine, Gottlieb, & Martin, 2003). The Spinach aptamer starts with a single G nucleotide, but for riboswitch–Spinach fusion constructs that do not start with a G, one should add 1–2 G nucleotides immediately after the TATA in the promoter sequence.
- If interested in testing alternate transducer stems, design constructs that include altered lengths of the natural riboswitch P1 stem.
- Another DFHBI-binding aptamer sequence, called Spinach2, has been developed that demonstrates enhanced folding stability and brightness at 37 °C (Strack, Disney, & Jaffrey, 2013). To use Spinach2, simply replace the Spinach sequence in the biosensor design (nucleotide changes are bold underlined).

WT Vc2-Spinach2 sequence: 5′-GA**T**G**TA**AC**T**GAATGAAATGGTGA AGGACGGGTCCAcacgcacagggcaaaccattcgaaa gagtgggacgcaaagcctccggcc taaaccagaagacatggtaggtagcggggttaccgatgTTGTTGAGTAGAGTGTGAGC TCCGTAACT**A**GT**TA**C**A**TC-3′

2.1.1 Equipment and materials

PCR thermal cycler
Agarose gel electrophoresis equipment
Phusion polymerase with HF buffer
dNTPs
Gel extraction or PCR clean-up kit

2.1.2 Procedure for generating DNA template for transcription

To prepare a 100-μL PCR reaction, combine the following in a thin-walled PCR tube:

20 μL 5 × Phusion HF buffer
10 μL 2 m*M* each dNTP
1 μL 40 μ*M* forward primer
1 μL 40 μ*M* reverse primer
1 μL 10–100 ng DNA template
76 μL ddH_2O
1 μL Phusion HF polymerase (add last)

The following is a standard thermocycler protocol that has been used for the WT Vc2-Spinach construct: Initial denaturation 98 °C for 1 min; 35 cycles of 98 °C 5–10 s, 66 °C for 20 s, 72 °C for 15 s, final extension 72 °C for 5 min. After the reaction, an aliquot of the PCR reaction is analyzed on an agarose gel. The PCR products are purified either via commercial gel extraction or PCR clean-up kits following the manufacturer's protocol and the product can be eluted in ddH_2O or the provided elution buffer.

Notes and tips:

– The NEB Website has a web form for estimating annealing temperatures for Phusion reactions (https://www.neb.com/tools-and-resources/interactive-tools/tm-calculator).

2.2. Preparation of RNAs by *in vitro* transcription

This step describes how to transcribe RNA *in vitro*, purify the RNA by polyacrylamide gel electrophoresis (PAGE), and accurately quantify the RNA concentration using a hydrolysis assay (Wilson, Cohen, Wang, & Hammond, 2014) (Estimated duration: 1 day).

2.2.1 Materials and equipment

UV/Vis spectrophotometer
Heat block (37, 70, 95 °C)
Polyacrylamide gel electrophoresis equipment

UV fluorescent TLC plate
Short wave UV light source
Razor blades
Microcentrifuge tube rotator
National Diagnostics Sequagel UreaGel system
Inorganic phosphatase
T7 RNA polymerase (NEB, 50 U/μL)
Ammonium persulfate (APS)
Tetramethylethylenediamine (TEMED)

Buffers and solutions that can be prepared in advance are listed here, whereas solutions that should be freshly prepared are described in the procedure. Prepare all solutions using ddH_2O water.

10× Transcription buffer

Component	Final concentration (m*M*)	Stock	Amount
Tris–HCl, pH 8.0	400	1 *M*	4 mL
$MgCl_2$	200	1 *M*	2 mL
DTT	100	1 *M*	1 mL
Spermidine	20	500 m*M*	400 μL

Add water to 10 mL, sterile filter, store at −20 °C.

Crush soak buffer

Component	Final concentration (m*M*)	Stock (*M*)	Amount (mL)
Tris–HCl, pH 7.5	10	1	10
NaCl	200	5	40
EDTA, pH 8.0	1	0.5	2

Add water to 1 L, autoclave, store at RT.

10× Tris–Borate–EDTA (TBE) buffer

Component	Final concentration (m*M*)	Stock	Amount
Tris	900	–	108.99 g
Boric acid	900	–	55.64 g
EDTA, pH 8.0	10	0.5 *M*	20 mL

Adjust pH to 8.3, add water to 1 L, sterile filter, store at RT.

2× Urea gel loading buffer (ULB)

Component	Final concentration	Stock	Amount
Sucrose	20%	–	2 g
Bromophenol Blue	0.05%	–	5 mg
Xylene cyanol	0.05%	–	5 mg
SDS	0.1%	10%	100 µL
TBE	1×	10×	1 mL
Urea	~18 *M*	–	11 g

Add water to 10 mL, stir for 2 h at room temperature, decant the solution from any undissolved urea, filter to sterilize, and store at 4 °C.

1× Tris–EDTA (TE) buffer

Component	Final concentration (m*M*)	Stock (*M*)	Amount (µL)
Tris–HCl, pH 7.5	10	1	100
EDTA, pH 8.0	1	0.5	20

Add water to 10 mL, sterile filter, store at RT.

Inorganic pyrophosphatase solution

Component	Final concentration	Stock	Amount
Glycerol	25%	50%	100 µL
HEPES–KOH, pH 7.5	50 m*M*	1 *M*	10 µL
DTT	0.5 m*M*	200 m*M*	0.5 µL
H_2O	–	–	89.5 µL
Inorganic pyrophosphatase	0.5 U/µL	–	100 U

Aliquot and store at −20 °C.

10× Sodium carbonate buffer

Component	Final concentration	Stock	Amount
Na_2CO_3	500 m*M*	–	0.53 g
EDTA	10 m*M*	0.5 *M*	200 µL

Adjust pH to 7.0, add water to 10 mL, sterile filter, store at −20 °C.

2.2.2 Procedures

Step 1: Transcription of RNA (estimated duration: 3 h)

1.1 To prepare a 50 μL transcription reaction, combine the following in a 1.5 mL microcentrifuge tube:
- 5 μL 10 × transcription buffer
- 8 μL 25 m*M* rNTPs
- 1–32 μL DNA template (1 μg)
- 1 μL inorganic pyrophosphatase
- ddH_2O to 46 μL
- 4 μL T7 RNA polymerase (add last)

1.2 Incubate reaction at 37 °C for 3 h.

1.3 Quench the reaction with 50 μL 2 × ULB. Place on ice if not immediately loading on the gel for purification. It is also possible to store the RNA overnight at −20 °C until ready to purify by PAGE.

Step 2: Purification of RNA (estimated duration: 4 h to 1 day)

2.1 To purify the desired RNA product away from abortive transcripts and unreacted NTPs, first prepare a 6% polyacrylamide gel. For sample volumes of 100–200 μL, we prepare a 28 cm long by 16.5 cm wide and 1.5 mm thick gel with eight sample lanes. Set up the electrophoresis equipment and fill buffer reservoirs with 1 × TBE buffer.

2.2 Load quenched reaction on polyacrylamide gel using a narrow-tip pipette and run the gel at 25 W. Run for ~1.5 h, or until the RNA has migrated 1/3–1/2 of the distance of the gel as indicated by the distance traveled by the xylene cyanol and bromophenol blue dyes, which migrate similar to 106 and 26 nt length oligonucleotides, respectively (Sambrook, Fritsch, & Maniatis, 1989).

2.3 To isolate the RNA, first peel off one glass plate from the gel and cover the exposed side with plastic wrap. If more than one RNA is being purified, be sure to label the order of the lanes at this time on the plastic wrap. Flip the gel and repeat for the other side, so that the gel is encased in plastic wrap.

2.4 To visualize the RNA by UV shadowing, place the wrapped gel on a fluorescent TLC plate and hold a UV light over the gel in a dark location. While the TLC plate should fluoresce, nucleotides absorb UV light and are revealed as dark, nonfluorescent bands in the gel. Typically the amount of DNA template used is too low to be visualized by this method, but RNA and NTPs are visible. Quickly mark the location of the RNA band, either by outlining the band or marking dots on the

four corners of the band, with a marker to minimize RNA damage from UV exposure.

2.5 Using a clean razor blade or other cutting utensil, excise the outlined RNA band, transfer it to a fresh glass plate, and dice the gel piece into ~1 mm cubes. Transfer the diced gel pieces into a fresh 1.5 mL microcentrifuge tube and add 450 μL crush soak buffer.

2.6 To extract the RNA, incubate gel pieces in crush soak buffer on a rotator for either 2 h at RT or overnight at 4 °C.

Step 3: Precipitation of RNA (estimated duration: 2 h)

3.1 To collect the RNA that has been extracted by the crush soak solution, centrifuge the sample briefly at 4500 rcf for 30 s at 4 °C then transfer the supernatant into a fresh 1.5 mL microcentrifuge tube. Use a narrow-tip pipette to avoid transferring any gel pieces. To precipitate the RNA, add 1 mL of ice-cold 100% ethanol. Mix the solution well by inverting the tube and store at −20 °C for at least 1 h.

3.2 Pellet the RNA by spinning at 13,200 rpm for 20 min at 4 °C in a microcentrifuge. Decant or remove the supernatant using a micropipettor and let the RNA pellet dry in open air for ~30 min or until all ethanol has evaporated.

3.3 Resuspend the pellet in 30 μL 1 × TE buffer. For a transcription reaction as described in Step 2, this typically results in an RNA solution of ~10 μ*M* concentration. Aliquot the RNA solution for storage at −20 °C to prevent degradation from multiple freeze-thaw cycles.

Step 4: Quantification of RNA concentration (estimated duration: 2 h)

4.1 Determine the approximate RNA concentration by measuring the A_{260} on a UV/Vis spectrophotometer. For structured RNAs like riboswitches and these biosensor constructs, the nearest-neighbor method (Kallansrud & Ward, 1996) overestimates the extinction coefficients because it does not take into account hypochromicity from base-pairing (Wilson et al., 2014).

4.2 To determine the accurate RNA concentration, perform the neutral pH thermal hydrolysis assay (Wilson et al., 2014). In this method, the RNA is hydrolyzed to individual NMPs, which eliminates the hypochromic effect, then the extinction coefficient is calculated as a summation of the extinction coefficients of NMPs in proportion to their representation in the sequence. Briefly, dilute an aliquot of the RNA stock to a starting A_{260} ~ 10 a.u. and prepare the following reaction in a 0.5-mL thin-walled microcentrifuge tube:

2 μL diluted RNA stock
2 μL 10× Na_2CO_3 buffer
16 μL ddH_20

Incubate the reaction at 95 °C for 1.5 h, cool to RT. Measure the A_{260} with a UV/Vis spectrophotometer and calculate the RNA concentration of the reaction using the following formula:

$$c_{\mathrm{RNA}} = \frac{A_{260,\mathrm{hydrolyzed}}}{b \sum_{i}^{\mathrm{A,C,U,G}} n_i \varepsilon_{260,i}}$$

where c is the RNA concentration, b is the path length, i is the nucleotide identity (A, C, G, or U), n_i is the frequency of nucleotide i present in the sequence, and ε_i is the molar extinction coefficient of the nucleotide i (Wilson et al., 2014). Multiply by the dilution factor to determine the stock RNA concentration. For a more detailed protocol, please see the methods paper by Wilson et al.

Notes and tips:

- To prevent RNAse contamination, autoclave or filter sterilize all solutions using a 0.22 μm filter. Keep samples containing RNA on ice to prevent breakdown. Use filter tips when pipetting RNA samples to avoid contamination.
- In Step 1.1, multiple transcription reactions can be set up in parallel, only limited by the quantity that can be purified on the PAGE gel. The reactions can be scaled up in quantity, depending on researcher needs or problems with transcription yield.
- If too many RNAs are being screened to purify by PAGE, it is possible to use a commercial RNA clean-up kit, such as the RNA Clean & Concentrator kit (Zymo Research). In this case, do not quench the reaction with ULB as described in Step 1.3. Replace Steps 2 and 3 with the manufacturer's protocol. However, it is advisable to check for abortive transcripts and to confirm the purity of the RNA through other means, such as denaturing agarose gel electrophoresis.
- In Step 2.1, to reduce band distortion and to remove unreacted ammonium persulfate from the gel, pre-run the gel for ~30 min prior to sample loading.
- In Step 2.2, to prevent contamination between samples, it is advisable to load samples in every other lane.
- In Step 2.5, to avoid contamination, do not reuse the cutting implement between different RNA samples.

– In Step 2.5, the efficiency of RNA extraction from the gel pieces may be increased by performing one freeze-thaw cycle prior to addition of crush soak buffer.

3. DETERMINATION OF LIGAND SELECTIVITY AND AFFINITY OF BIOSENSOR BY FLUORESCENCE ACTIVATION

A key functional test of any designed biosensor is whether fluorescence activation is observed in the presence of the ligand versus without ligand. Furthermore, fluorescence must be selectively turned on by the ligand of interest versus other related compounds that might be present in the cell. Finally, the apparent dissociation constant (K_d) of the biosensor–ligand complex should be within the physiologically relevant range, if the purpose is to measure *in vivo* concentrations of a metabolite or signaling molecule. Note that the apparent K_d is a simplified estimate of ligand binding affinity since the biosensor has to bind both ligand and DFHBI to elicit fluorescence. For this reason, the measurements are performed with saturating concentrations of DFHBI. Since temperature and magnesium concentrations can have dramatic effects on the stabilities of RNA structures, we recommend measuring the ligand selectivity and K_d at 37 °C and 3 m*M* Mg^{2+}, which is closer to cellular conditions than standard assay conditions.

Nevertheless, care needs to be taken in terms of interpreting these *in vitro* results as being fully reflective of cellular fluorescence measurements. While the maximum fluorescence activation *in vitro* for a given biosensor generally corresponds to the signal-to-noise observed *in vivo*, the *in vitro* measurement is performed under conditions such that the biosensor is at limiting concentrations relative to ligand, which is not always the case in the cell. In addition, the *in vitro* binding buffer is an inadequate proxy for the complex cellular milieu, so the binding affinity of the biosensor *in vivo* may be different. Nevertheless, we have found that the selectivity and relative binding affinities of different biosensor constructs *in vivo* are accurately reflected by the *in vitro* measurements (Kellenberger et al., 2013).

3.1. Determination of ligand selectivity

3.1.1 Additional materials and equipment

Fluorescent plate reader with heating control
Corning Costar 96-well black plate
c-di-GMP (Axxora, LLC; Farmingdale, NY)
Other ligand analogs for selectivity experiments.

Buffers and solutions that can be prepared in advance are listed here, whereas solutions that should be freshly prepared are described in the procedure. Prepare all solutions using ddH_2O water.

2× Renaturing buffer

Component	Final concentration (m*M*)	Stock (*M*)	Amount
HEPES–KOH, pH 7.5	80	1	800 μL
$MgCl_2$	6	1	60 μL
KCl	250	2	1.25 mL

Add water to 10 mL, sterile filter, store at RT.

DFHBI stock (Lucerna; New York, NY)

Component	Final concentration	Stock	Amount
DFHBI	20 m*M*	–	1 mg
DMSO	–	–	198 μL

Aliquot in amber tubes and store at −20 °C.

3.1.2 Procedures

Step 5: Preparation of binding reaction components (estimated duration: 20 min)

5.1 To determine the fluorescent activation of the biosensor for different ligands, prepare 1 m*M* ligand stocks (e.g., c-di-GMP) for a final concentration of 100 μ*M* in the binding reactions.

5.2 To prepare the binding reactions, first a master mix containing buffer components and DFHBI is freshly prepared:

4 μL 1 *M* HEPES, pH 7.5
6.25 μL 2 *M* KCl
0.3 μL 1 *M* $MgCl_2$
10 μL 100 μ*M* DFHBI
59.45 μL ddH_2O.

For analyzing additional or replicate samples, multiply these volumes by the number of samples plus one. The master mix given above accounts for 80 μL of the 100 μL final reaction volume, in which the remaining volume will be from RNA and ligand solutions added separately. The final concentration of components of the binding reaction

will be 40 m*M* HEPES, pH 7.5, 125 m*M* KCl, 3 m*M* $MgCl_2$, 10 μ*M* DFHBI, 100 n*M* RNA, and 100 μ*M* ligand.

5.3 To renature the RNA biosensor before addition to the binding reaction, prepare a 2 μ*M* stock solution of RNA by diluting in ddH_2O, then add an equal volume of 2 × renaturation buffer to make a 1 μ*M* stock solution of RNA in renaturation buffer. Prepare enough volume to add 10 μL of this RNA solution to each binding reaction for a final concentration of 100 n*M*. Heat the renaturation reaction to 70 °C for 3 min, then cool at ambient temperature for 5 min.

Step 6: Perform binding reactions and measure fluorescence activation (estimated duration: 2.5 h)

6.1 Add 10 μL of ligand stock or ddH_2O for the "no ligand" control to 1–3 replicate wells in a 96-well black plate followed by 80 μL master mix solution. A multichannel pipet may be used; however, do not reuse tips between different wells to prevent cross-contamination. To compare the sample fluorescence to the background fluorescence of DFHBI without RNA, prepare 1–3 replicate wells with 20 μL ddH_2O + 80 μL master mix.

6.2 Add 10 μL renatured RNA to each binding reaction, excluding "no RNA" control. Mix reactions well and check that there are no bubbles in or on the side of each well. To remove any bubbles, either use a sterile pipet tip to pop or mix with the solution, or centrifuge the plate in a microcentrifuge equipped with a 96-well plate holder for 30 s at 500 rcf.

6.3 To measure fluorescence, load the plate in a fluorescence plate reader and monitor fluorescence using the following parameters:

Excitation: 460 nm
Emission: 500 nm
Total time: 2 h
Read time: every 5 min
Temperature: constant 37 °C

6.4 To analyze the data, choose a time point where the fluorescence reading has equilibrated. To determine ligand selectivity, graph fluorescence intensity for each ligand versus no ligand control, subtracting from each sample the background fluorescence of an empty well or a well containing buffer without DFHBI, which have approximately equal fluorescence. We do not subtract the fluorescence of DFHBI without RNA, as this represents the real background in the experiment

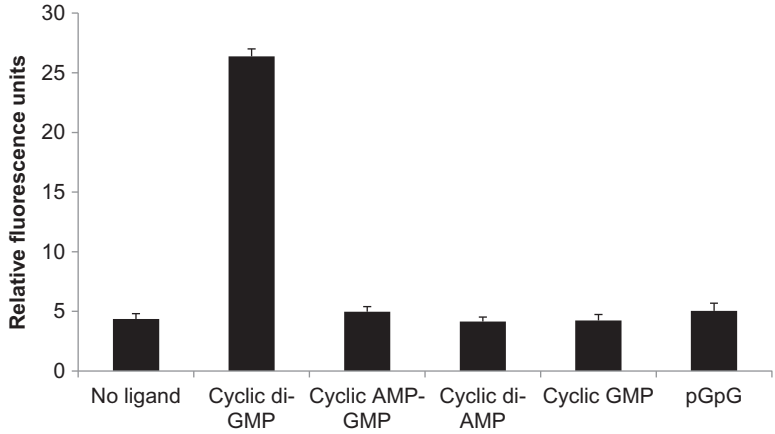

Figure 2 Selectivity measurements for the WT Vc2-Spinach aptamer. The WT Vc2-Spinach aptamer demonstrates selectivity for cyclic di-GMP. WT Vc2-Spinach (100 n*M*) elicits fluorescence from DFHBI (10 μ*M*) selectively in response to cyclic di-GMP (100 μ*M*) but not other related ligands (100 μ*M*). Error bars represent the standard deviation between three independent replicates carried out at 37 °C, 3 m*M* Mg^{2+}.

and would misrepresent the fold activation of the biosensor. Representative results for the Vc2-Spinach biosensor are shown in Fig. 2.

Notes and tips:

- In the procedure described, a single high concentration (100 μ*M*) of ligands is analyzed. If the biosensor has a very low dissociation constant (K_d) for binding its cognate ligand (high affinity), it may appear to have a fluorescence response to other ligands. In case this is observed, an intermediate (e.g., 50-fold lower) versus high ligand concentration may be tested, or determination of ligand affinity should be performed following the procedure described below.
- It is suggested that at least two technical replicates should be performed for each sample on a plate to account for systematic error. Furthermore, at least two independent replicates of the experiment should be performed to account for sample variability. Independent experiments should be prepared from different master mixes, although preparing samples on different days also helps account for other sources of variability.
- In Step 6.1, it is recommended to add ligand first before the master mix as this makes it easier to keep track of which samples have already been added. However, this order does require that micropipette tips be changed between additions of master mix to prevent contamination.

The reverse order, where master mix is added before ligand, is also fine and allows micropipette tips to be reused.

- In Step 6.3, fluorescence readings are measured at 37 °C and the binding reactions prepared in Step 5.2 contain 3 m*M* $MgCl_2$ in order to model biosensor activity under physiological conditions. For initial screening purposes, the binding reactions may be performed at room temperature with 10 m*M* $MgCl_2$ instead, which typically increases the fluorescence activation and ligand binding affinity of the biosensor.
- Use of higher concentrations of the RNA biosensor would also increase fluorescence signal. Thus, it is important to determine the RNA concentration accurately (see Step 4).
- If high background fluorescence is observed without ligand, one may consider modifying the construct to destabilize the transducer stem. To increase fluorescence activation, one may consider using the Spinach2 sequence or the alternate fluorescent dyes DFHBI-1T (Lucerna), which has an improved fluorescence excitation and emission profile (Song, Strack, Svensen, & Jaffrey, 2014; Strack et al., 2013).
- In Step 6.4, keep in mind that different biosensor constructs may demonstrate different binding kinetics. For example, Vc2-Spinach takes 40 min to fully equilibrate with ligand, but other biosensors display full activation within 5 min (Kellenberger et al., 2013, unpublished results).

3.2. Determination of ligand affinity

A protocol similar to the one described above for determination of ligand selectivity (Steps 5 and 6) can be used to measure the affinity of the biosensor for a specific ligand, with the following changes:

1. The RNA biosensor concentration must be below the expected K_d to measure the dissociation constant accurately (Hall & Kranz, 2007). Thus, we typically use a concentration of RNA that is approximately 10-fold lower than the expected K_d. For example, in Step 5.3, the final concentration for the 30 n*M* (Kd $\sim 230 \pm 50$ nM). It is advisable to determine the lower limit of detection for the fluorescence activation over background.
2. In Step 5.1, in place of different ligand solutions, use different concentrations of the ligand of interest that span both sides of the expected K_d value to obtain a full binding curve. For example, to determine the binding affinity of WT Vc2-Spinach to c-di-GMP, a no ligand control and 12 serial dilutions of c-di-GMP were made that ranged from final concentrations of 100 μ*M* to 100 p*M* in ~3-fold increments.

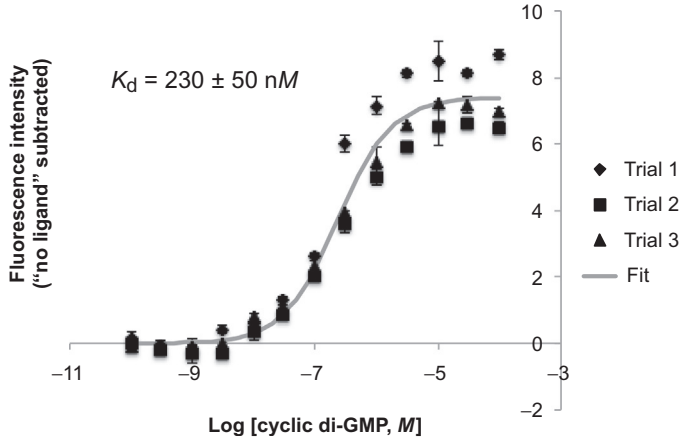

Figure 3 Affinity measurement of the WT Vc2-Spinach aptamer for c-di-GMP. WT Vc2-Spinach (30 n*M*) binds cyclic di-GMP with an apparent K_d of 230 ± 50 n*M* in the presence of saturating DFHBI (10 μ*M*). Trials from three independent replicates of experiments at 37 °C and 3 m*M* Mg^{2+} are shown.

3. Compile the fluorescence intensity for each ligand concentration from a timepoint where binding has equilibrated. Subtract the background of the "no ligand" sample from each data point and fit the data to an appropriate binding model using an Excel spreadsheet or commercial software to calculate the K_d value. At least three independent replicates of the experiment should be performed to account for sample variability, and the average K_d value with standard deviation is reported. Representative results for the Vc2-Spinach biosensor are shown in Fig. 3.

4. DETERMINATION OF BINDING KINETICS OF BIOSENSOR

For a fluorescent biosensor to be used to study the dynamics of a target metabolite or signaling molecule, it must respond within the time range of the cellular process being studied. Furthermore, it is important to characterize both ligand association and dissociation, as one advantage of fluorescent biosensors versus reporter systems is the ability to monitor signal production and breakdown dynamically. Again, we recommend measuring binding kinetics at 37 °C and 3 m*M* Mg^{2+} to mimic physiological conditions. The measured activation and deactivation rates will also depend on the concentrations of the biosensor, ligand of interest, and DFHBI.

Since fluorescence activation involves a ternary complex, one may not easily derive rate constants for the individual steps from the kinetic experiments described. Our proposed binding model assumes DFHBI can only bind to a specific conformation of the biosensor (RNA*) that is normally higher in energy than the ground-state conformation (Fig. 4). Thus, background fluorescence is low because there is very little partitioning to the RNA*•DFHBI complex. For example, we observe <5% fluorescence change for Vc2-Spinach + DFHBI versus DFHBI alone samples, which implies that very little RNA*•DFHBI is present under these conditions. Upon addition of ligand, ligand binding favors the conformational change to RNA*•L, which results in fluorescence activation of DFHBI by formation of the ternary complex. This model does not take into account the *cis* and *trans* forms of DFHBI, which have been shown to bind Spinach with slightly different affinities and to be thermally reversible (Wang et al., 2013).

For the activation experiments described below, the biosensor is added directly to a mixture of cyclic di-GMP and excess DFHBI (10 μ*M*). Because the amount of RNA*•DFHBI is low, presumably we are observing the combined steps of ligand binding, RNA conformational change, and

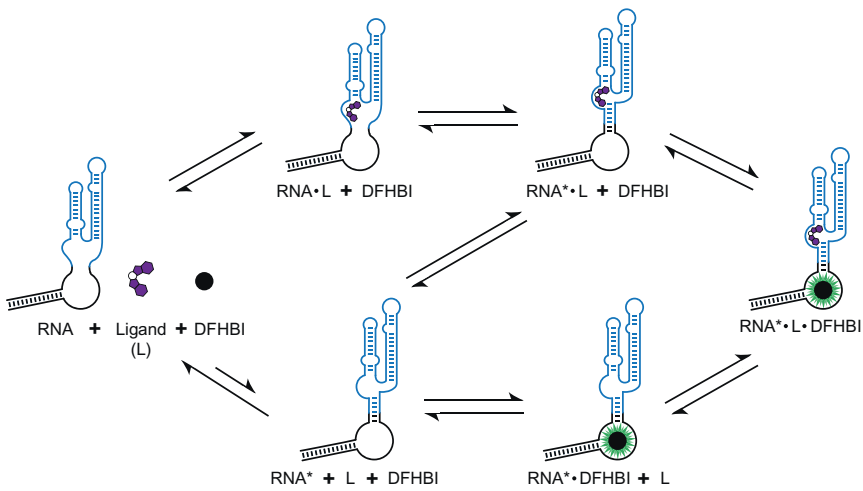

Figure 4 Association of RNA, ligand, and DFHBI to form the fluorescent ternary complex. Before the stable ternary complex forms, ligand binds to the RNA, RNA presumably undergoes a conformational change to RNA*, and DFHBI binds to RNA*. Predicted pathways of complex formation are depicted.

DFHBI binding to form the fluorescent ternary complex. For the deactivation experiments described below, the RNA is first equilibrated with excess ligand and DFHBI to form the fluorescent ternary complex RNA*•L•DFHBI. The binding reaction then is split and passed through two gel fitration columns that have been preequilibrated with either buffer containing DFHBI + ligand or DFHBI alone. In the latter sample, absence of free ligand in the reaction solution should move the overall equilibrium to the left, although the kinetic parameters are complex and reflect two separate pathways for fluorescence deactivation.

4.1. Determination of activation rate

A protocol similar to the one described above for determination of ligand selectivity (Steps 5 and 6) can be used to measure biosensor binding kinetics. Begin by preparing the same ligand dilution and master mix as in Steps 5.1 and 5.2. To avoid fluctuations in fluorescence due to temperature, the most important aspect of the activation experiment is that the ligand/master mix and RNA solutions are preequilibrated at 37 °C before fluorescence measurement. To ensure that all solutions are warmed to 37 °C and to minimize RNA degradation, first carry out Step 6.1 followed by Step 5.3 with the modifications below, then proceed with the remaining steps in order.

1. In Step 6.1, after addition of ligand or ddH_2O for "no ligand" sample to the reaction, prewarm the solution to 37 °C in the plate reader.
2. In Step 5.3, after preparing the 1 μ*M* stock solution of RNA in renaturation buffer, heat the RNA to 70 °C for 3 min, then incubate at 37 °C until added to the plate. To minimize RNA degradation, do not perform this step until the reaction solutions containing DFHBI and ligand are equilibrated at 37 °C.
3. In Step 6.2, add 10 μL RNA to each binding reaction using a multichannel pipettor, and mix reactions quickly by pipetting up and down. Immediately proceed to the next step, which is taking fluorescence readings.
4. In Step 6.3, use the following parameters:
   Excitation: 460 nm
   Emission: 500 nm
   Total time: 2 h
   Read time: every 1–2 min
   Temperature: constant 37 °C

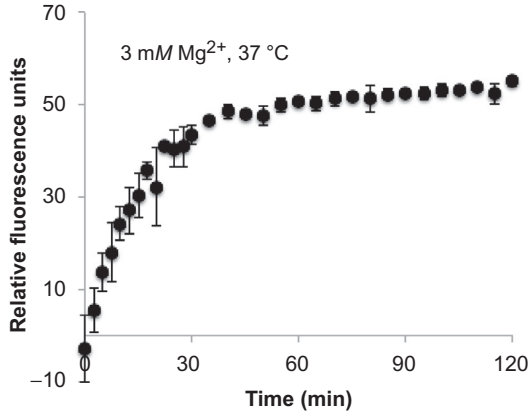

Figure 5 Measurement of the activation rate of WT Vc2-Spinach. WT Vc2-Spinach (100 n*M*) fully binds to DFHBI (10 μ*M*) and cyclic di-GMP (100 μ*M*) within 40 min at 37 °C and 3 m*M* Mg^{2+}. Error bars represent the standard deviation of two replicate samples.

5. In Step 6.4, plot the fluorescence intensity over time to determine time required for full ligand association. Representative results for the Vc-Spinach biosensor are shown in Fig. 5.

Notes and tips:

– If unexpected fluorescence variation is observed in the first few minutes, this is likely due to fluctuation in temperature from the sample. Ensure that both the RNA and the ligand mixture are preequilibrated at 37 °C before mixing.

4.2. Determination of deactivation rate

To perform the deactivation experiment, free ligand in the sample will be removed by passing half of an equilibrated binding reaction through a size-exclusion column that has been preloaded with DFHBI and buffer but no c-di-GMP. The free c-di-GMP in the sample will be retained by the column, whereas ligand-bound RNA and free RNA, should elute from the column along with preloaded DFHBI and buffer. As a control, the other half of the sample is passed through a size-exclusion column that has been preloaded with c-di-GMP, DFHBI, and buffer.

4.2.1 Additional materials and equipment

G-25 columns.

4.2.2 Procedure

Step 7: Preparation of binding reaction (estimated duration: 1.5 h)

7.1 Prepare the following two exchange buffers, one with and the other without ligand, and prewarm the solution to 37 °C. The amounts shown should be sufficient for 3 column volumes each (~1.5 mL).

60 μL 1 *M* HEPES, pH 7.5
93.75 μL 2 *M* KCl
4.5 μL 1 *M* $MgCl_2$
150 μL 100 μ*M* DFHBI
150 μL 1 m*M* ligand (e.g., c-di-GMP) or ddH_2O
1041.75 μL ddH_2O

7.2 Renature the RNA before adding to the binding reaction by preparing a 2 μ*M* stock solution of RNA in ddH_2O, then add an equal volume of 2 × renaturation buffer to make a 1 μ*M* stock solution of RNA in renaturation buffer. Prepare enough volume to add 21 μL of this RNA solution to each binding reaction for a final concentration of 100 n*M*. Heat the renaturation reaction to 70 °C for 3 min, then cool at ambient temperature for 5 min.

7.3 Prepare the following binding reaction (210 μL total volume) in one well of a black 96-well plate:

8.4 μL 1 *M* HEPES, pH 7.5
13.13 μL 2 *M* KCl
0.63 μL 1 *M* $MgCl_2$
21 μL 100 μ*M* DFHBI
21 μL 1 m*M* ligand (e.g., c-di-GMP)
21 μL 1 μ*M* renatured WT Vc2-Spinach RNA
124.84 μL ddH_2O

The final concentrations of components of the binding reaction are 40 m*M* HEPES, pH 7.5, 125 m*M* KCl, 3 m*M* $MgCl_2$, 10 μ*M* DFHBI, 100 n*M* RNA, and 100 μ*M* ligand.

7.4 Incubate the reaction at 37 °C in the fluorescent plate reader. Monitor fluorescence using the parameters given in Step 6.3 until the reaction has fully equilibrated. For the sample with Vc2-Spinach, this takes approximately 1 h.

Step 8: Perform buffer exchange and measure fluorescence deactivation (estimated duration: 2 h)

8.1 First prepare two G-25 spin columns, one equilibrated with ligand and one without, by saturating each of the columns with 3 column volumes (~500 μL) of the exchange buffer. Briefly, load 500 μL buffer on each

column and centrifuge the spin columns at 730 rcf for 1 min at 37 °C. Discard the flow through and repeat this buffer exchange two more times. Place the column onto a clean collection tube.

8.2 Once the binding reaction from Step 7.4 is equilibrated and the G-25 columns are prepared, immediately load 100 μL of the binding reaction onto each of the G-25 spin columns and centrifuge at 730 rcf for 1 min to elute. Due to size exclusion, the ligand-bound RNA and RNA will flow through, whereas the free ligand will be retarded in the gel matrix.

8.3 Immediately transfer each of the flow-through solutions to clean and separate wells of the 96-well plate and record fluorescence with the following parameters:

Excitation: 460 nm
Emission: 500 nm
Total time: 2 h
Read time: every 1–2 min
Temperature: constant 37 °C

8.4 To analyze the data, plot the fluorescence intensity over time for each sample. Representative results are shown in Fig. 6.

Notes and tips:

– The manufacturer notes that G-25 column should not be left dry for a long period of time. Proceed directly from Step 8.1 to 8.2.

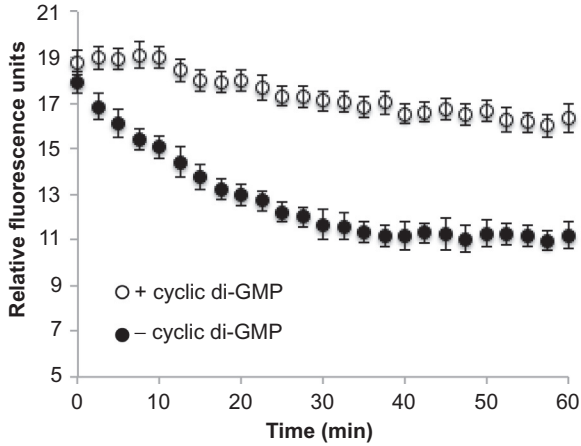

Figure 6 Measurement of the deactivation rate of WT Vc2-Spinach. WT Vc2-Spinach RNA (100 n*M*) fully deactivates in response to loss of ligand within 40 min at 37 °C and 3 m*M* Mg^{2+}. Some loss of ligand is observed in the sample passed through a column buffer exchanged with cyclic di-GMP, indicating retention of cyclic di-GMP or DFHBI on the column. Error bars represent the standard deviation of two replicate samples.

- Steps 8.2 and 8.3 should be performed quickly. If the deactivation rate is too fast or if the above procedure proves problematic, consider other strategies to examine the deactivation of the biosensor, such as enzymatic degradation of the metabolite or favoring ligand dissociation by dilution.
- To avoid fluctuations in fluorescence due to differential elution and retention between the two columns, consider eluting the RNA sample into another 100 μL of the exchange buffer in Step 8.2. This helps to ensure that the buffer components stay the same between samples.
- If possible, use a saturating concentration of ligand in the binding reaction and an excess of ligand when equilibrating the spin column. This will reduce variations in fluorescence due to small amounts of ligand retained in the column.

REFERENCES

Blount, K. F., Wang, J. X., Lim, J., Sudarsan, N., & Breaker, R. R. (2007). Antibacterial lysine analogs that target lysine riboswitches. *Nature Chemical Biology*, *3*, 44–49.

Cruse, W. B., Saludjian, P., Biala, E., Strazewski, P., Prangé, T., & Kennard, O. (1994). Structure of a mispaired RNA double helix at 1.6-Å resolution and implications for the prediction of RNA secondary structure. *Proceedings of National Academy of Sciences of United States of America*, *91*, 4160–4164.

Deigan Warner, K., Chen, M. C., Song, W., Strack, R. L., Thorn, A., Jaffrey, S. R., et al. (2014). Structural basis for activity of highly efficient RNA mimics of green fluorescent protein. *Nature Structural and Molecular Biology*, *21*, 658–663.

Domaille, D. W., Zeng, L., & Chang, C. J. (2010). Visualizing ascorbate-triggered release of labile copper within living cells using a ratiometric fluorescent sensor. *Journal of the American Chemical Society*, *132*, 1194–1195.

Fowler, C. C., Brown, E. D., & Li, Y. (2010). Using a riboswitch sensor to examine coenzyme B12 metabolism and transport in *E. coli*. *Chemistry & Biology*, *17*, 756–765.

Hall, K. B., & Kranz, J. K. (2007). Nitrocellulose filter binding for determination of dissociation constants. *Methods in Molecular Biology*, *118*, 1–10.

Huang, H., Suslov, N. B., Li, N.-S., Shelke, S. A., Evans, M. E., Koldobskaya, Y., et al. (2014). A G-quadruplex-containing RNA activates fluorescence in a GFP-like fluorophore. *Nature Chemical Biology*, *10*, 686–691.

Kallansrud, G., & Ward, B. (1996). A comparison of measured and calculated single- and double-stranded oligodeoxynucleotide extinction coefficients. *Analytical Biochemistry*, *236*, 134–138.

Kellenberger, C. A., Wilson, S. C., Sales-Lee, J., & Hammond, M. C. (2013). RNA-based fluorescent biosensors for live cell imaging of second messengers cyclic di-GMP and cyclic AMP-GMP. *Journal of the American Chemical Society*, *135*, 4906–4909.

Kuzmine, I., Gottlieb, P. A., & Martin, C. T. (2003). Binding of the priming nucleotide in the initiation of transcription by T7 RNA polymerase. *The Journal of Biological Chemistry*, *278*, 2819–2823.

Liu, Z., He, W., & Guo, Z. (2013). Metal coordination in photoluminescent sensing. *Chemical Society Reviews*, *42*, 1568–1600.

Mandal, M., & Breaker, R. R. (2004). Adenine riboswitches and gene activation by disruption of a transcription terminator. *Nature Structural & Molecular Biology*, *11*, 29–35.

Nakayama, S., Luo, Y., Zhou, J., Dayie, T. K., & Sintim, H. O. (2012). Nanomolar fluorescent detection of c-di-GMP using a modular aptamer strategy. *Chemical Communications*, *48*, 9059–9061.

Nelson, M. D., & Fitch, D. H. A. (2011). Molecular methods for evolutionary genetics. In V. Orgogozo, & M. V. Rockman (Eds.), *Vol. 772. Methods in molecular biology* (pp. 459–470). New York, NY: Humana Press.

Newman, R. H., Fosbrink, M. D., & Zhang, J. (2011). Genetically encodable fluorescent biosensors for tracking signaling dynamics in living cells. *Chemical Reviews*, *111*, 3614–3666.

Paige, J. S., Nguyen-Duc, T., Song, W., & Jaffrey, S. R. (2012). Fluorescence imaging of cellular metabolites with RNA. *Science*, *335*, 1194.

Paige, J. S., Wu, K. Y., & Jaffrey, S. R. (2011). RNA mimics of green fluorescent protein. *Science*, *333*, 642–646.

Römling, U., Galperin, M. Y., & Gomelsky, M. (2013). Cyclic di-GMP: The first 25 years of a universal bacterial second messenger. *Microbiology and Molecular Biology Reviews*, *77*, 1–52.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Serganov, A., & Nudler, E. (2013). A decade of riboswitches. *Cell*, *152*, 17–24.

Song, W., Strack, R. L., Svensen, N., & Jaffrey, S. R. (2014). Plug-and-play fluorophores extend the spectral properties of Spinach. *Journal of the American Chemical Society*, *136*, 22–25.

Strack, R. L., Disney, M. D., & Jaffrey, S. R. (2013). A superfolding Spinach2 reveals the dynamic nature of trinucleotide repeat-containing RNA. *Nature Methods*, *10*, 1219–1224.

Wang, P., Querard, J., Maurin, S., Nath, S. S., Le Saux, T., Gautier, A., et al. (2013). Photochemical properties of Spinach and its use in selective imaging. *Chemical Science*, *4*, 2865–2873.

Wilson, S. C., Cohen, D. T., Wang, X. C., & Hammond, M. C. (2014). A neutral pH thermal hydrolysis method for quantification of structured RNAs. *RNA*, *20*, 1153–1160.

Zhou, X., Herbst-Robinson, K. J., & Zhang, J. (2012). Visualizing dynamic activities of signaling enzymes using genetically encodable FRET-based biosensors from designs to applications. *Methods in Enzymology*, *504*, 317–340.