# **Supplementary Information**

*Bioluminescent stem-loop probes for highly sensitive nucleic acid detection* Eric A. Hunt, Sapna K. Deo

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# **Supplementary Information**

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# 1) Expression and purification of Renilla luciferase

The gene for the RLuc8 mutant<sup>1</sup> of the wild-type *Renilla* luciferase was cloned into the pBAD/*Myc*-HisA vector (Invitrogen) and transformed into *E. coli* LMG194 for expression. 200 mL cultures were grown at 37 °C to an OD<sub>595 nm</sub> of ~0.6, at which point expression was induced with 0.2 % L-arabinose. Expression was allowed to continue for 5 h at 37 °C. Cells were collected by centrifugation and lysed by sonicating (2 min, 5 s on/off pulse) in 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.2 buffer. Crude extract was purified by affinity chromatography using a Ni-NTA agarose solid phase. The protein was allowed to bind for 2 h at ambient temperatures before eluting with 500 mM imidazole. The purified RLuc8 was then concentrated using 10 kDa MWCO Millipore filters; bufferexchanged into a 100 mM sodium phosphate, 150 mM sodium chloride, pH 7.2 buffer; and stored at 4 °C. The purified RLuc8 is stable for several months.

# 2) Conjugation of the stem-loop probe

The custom oligonucleotide sequence:

5'[AminoC6]

<sup>↑</sup>GGGGGGA TCAACATCAGTCTGATAAGCTA [Bio-dT]CCCCC↓

[Dabcyl-Q]3'

was ordered from Eurofins MWG Operon. The 5' amino modification was chemically converted to a benzaldehyde moiety by succinimidyl 4-formylbenzoate (SFB, Pierce – Thermo Fisher Scientific Inc.) in 50x excess in a 50 mM sodium phosphate, 75 mM sodium chloride, pH 7.2 buffer. Amine residues of purified RLuc8 were modified with succinimidyl 4-hydrazinonicotinate acetone hydrazone (SANH, Pierce – Thermo Fisher Scientific Inc.) in 10x excess in a 100 mM sodium phosphate, 150 mM sodium chloride, pH 7.2 buffer. Once a stable amide bond is formed between 4-hydrazinonicotinate and RLuc8, the alkyl hydrazone is hydrolyzed by buffer exchanging into a 100 mM sodium citrate, 150 mM sodium chloride, pH 6.0 buffer, which yields the aldehyde-reactive hydrazine moiety. SFB-modified oligonucleotide was added in 1.5x excess to form a stable hydrazone conjugate (Figure S1).

A large molecule such as RLuc8 (39.5 kDa) is much bulkier than the typical organic dyes used in FSLP design. In initial designs for the BSLP, the steric effects of having a large molecule attached to the probe resulted in a very low  $T_m$  for the stem. This prevented the probe from closing tightly and properly quenching the bioluminescent signal, therefore a six base pair stem containing 83% GC pairs was used to increase the  $T_m$  to an adequate level. To remove the excess RLuc8 remaining in solution after conjugation (Figure S2), we incorporated a biotin affinity-handle on the probe stem.

It is also evident from the SDS-PAGE analysis that while the stoichiometry of the conjugation reaction is controlled to promote a 1:1 ratio of probe to RLuc8, the formation of polyconjugates is inevitable and quite prominent. An estimation of the average number of probe molecules attached to each RLuc8 was performed by quantifying the purified conjugate by BCA protein assay, and by the molar extinction



*Figure S1.* Schematic of chemical reaction for attaching the stem-loop probe to RLuc8. The SANH succinimidyl ester reacts with amine side chains on Lys and/or the N-terminus of the protein, and the SFB succinimidyl ester reacts with the 5' amino-modification on the stem-loop probe. Transfer of the SANH-RLuc8 intermediate to pH 6.0 citrate buffer hydrolyzes the alkyl hydrazone of SANH, yielding the hydrazine functionality which readily reacts with the benzaldehyde of the SFB-Probe intermediate. The final product is a stable covalent linkage between the two biomolecules.

coefficient for the DABCYL modification. By these two methods, there was an approximately 4:1 ratio of probe to RLuc8. While the BCA assay is not an extremely accurate or precise method of protein quantification, this does provide further evidence that polyconjugates are readily formed in the conjugation reaction. Due to the size of RLuc8, however, the formation of polyconjugates should not inhibit normal functionality of the BSLP. It may, however, cause higher order polyconjugates to be more effectively quenched than those containing only one or two DABCYL functionalities.



*Figure S2.* A 12.5% SDS-PAGE gel of the non-purified BSLP following RLuc8 conjugation, stained with Coomassie Blue. Lane 1: Fisher BioReagents EZ-Run Prestained Protein Marker 0-118 kDa; Lane 2: purified RLuc8 (control); Lane 3: BSLP non-purified conjugate. Note that lanes 2 and 3 are not concentration matched. The discernable bands above that of the monoconjugate in lane 3 represent polyconjugate BSLPs.

#### 3) Purification of the bioluminescent stem-loop probe

A purification column was prepared using 1 mL of monomeric avidin-immobilized agarose. The packed resin was washed with 8 mL of wash buffer (100 mM sodium phosphate, 150 mM sodium chloride, pH 7.0). Next, the column was washed with 6 mL of elution buffer (wash buffer containing 2 mM D-biotin) to block any irreversible biotin binding sites on the resin. The column was then regenerated with 12 mL of regeneration buffer (100 mM glycine, pH 2.8) followed by 8 mL of wash buffer. A 5 µL aliquot of the non-purified stem-loop probe was removed for comparison, and the remainder was added to the column and forced into the bed with 1 mL of wash buffer. The column was then sealed and incubated at ambient temperature for 1 h. To wash the probe, 1 mL aliquots of wash buffer were collected until the bioluminescent emission ( $\lambda_{max em.} = 485 \text{ nm}$ , Tecan GENios) resulting from the addition of the native substrate coelenterazine returned to baseline. The probe was then eluted with 200 µL aliquots of elution buffer until the absorbance of the 3' DABCYL modification ( $\lambda_{max abs.} =$ 475 nm,  $\epsilon_{475nm} \approx 30,000$ ) returned to baseline (Figure S3). Elution fractions were then collected based on their 475 nm absorption and subsequently pooled and concentrated using Millipore - Microcon YM-30 Centrifugal Filter Units. The column was washed with 4 mL of regeneration buffer and stored upright at 4 °C after washing with 5 mL of wash buffer containing 0.01% sodium azide.

Purification of the BSLP was necessary due to background noise caused by any unconjugated RLuc8. To address this issue, the thymidine in the stem of the probe was replaced with a biotin-modified thymidine (bio-T). This afforded a convenient affinity handle for purification without detriment to the structural integrity of the SLP sequence needed for proper stem-loop formation. The BSLP was then conjugated to RLuc8 using SANH/SFB chemistry and purified by affinity chromatography using an agarose solid phase modified with monomeric avidin. The monomeric avidin-biotin binding affinity is considerably lower than the typical avidin-biotin interaction (K<sub>d</sub> =  $10^{-8}$  M versus  $10^{-15}$ M) and biotinylated molecules may be displaced with biotin containing buffer. Purification effectively removed free RLuc8, lowering the residual bioluminescent background and allowing for an approximately 6-fold signal change to be seen between the fully open and closed conformations (Figure S4).



*Figure S3.* Purification of the stem-loop probe conjugate. RLuc8 elution was measured by total light bioluminescence in the presence of coelenterazine. Probe elution was monitored by the presence of the 3'DABCYL modification absorbing at 475 nm.

The fully purified BSLP is stored in 100 mM sodium phosphate, 1 mM EDTA, pH 7.4 at 4 °C, and is stable with only minor losses in activity for over 1 year. We have noticed no aggregation of the purified BSLP, but it was centrifuged for 1 min at 16,000 xg before every



*Figure S4.* The background noise caused by free RLuc8 is drastically reduced after purification. Without purification, signal at the upper and lower limits of the calibration is masked completely and no target can be detected.

use to ensure the removal of any insoluble matter. It should be noted that aggregation is unlikely due to the high charge density on the surface of the Rluc8 once functionalized with DNA.

#### 4) Assay setup

A standard white polypropylene 96-well plate was washed three times for 30 minutes at ambient temperature with 0.5% BSA in 100 mM sodium phosphate, 1 mM EDTA, pH 7.4 buffer. The plate was then rinsed and dried. Calibrations were performed using varying amounts of target mixed with 0.5 pmol of BSLP in a 100 mM sodium phosphate, 1 mM EDTA, pH 7.4 buffer and allowed to hybridize at ambient temperature for 3 h (hybridization for 1 h will also provide sufficient quantitative signal). Total-light bioluminescent readings were taken using a PerkinElmer Victor X Light bioluminometer equipped with a syringe auto-injector. The bioluminescent reaction was initiated by injecting 50  $\mu$ L of a 2.5:1000 dilution ( $\approx 6\mu$ M) of 1 mg/mL (in acidified methanol) native coelenterazine (NanoLight Technology) prepared in 100 mM sodium phosphate, 1 mM EDTA, pH 7.4 buffer immediately before use (1 mg/mL stock made in degassed, acidified ethanol and stored under argon at -80 °C) and shaking for 5 s. Total well volume including injected coelenterazine was



Figure S5. Initial calibrations run according to previous methods.<sup>2</sup>

held at 150  $\mu$ L. The amount of coelenterazine used does not have a noticeable effect on the background signal; therefore, the amount of coelenterazine injected was in excess of what the Rluc8 was able to process.

# 5) Stem-loop probe characterization

In order to compare the BSLP to a traditional FSLP, a FSLP using a 6-carboxyfluorescein (6FAM)/Black Hole Quencher 1 (BHQ1) fluorophore/quencher pair was ordered with the same sequence (minus the bio-T modification). The FSLP was characterized by fixing the target concentration at a point in the middle of the linear portion of the initial calibration curve (Figure S5, run according to previously published methods)<sup>2</sup> and varying the concentration of FSLP used to achieve the best S/N ratio (Figure S6 A-B, S7). The same procedure was also done for the BSLP (Figure S7). The optimal concentration for each SLP was chosen by varying the amount of SLP while holding the target concentration constant. The concentration at which the best S/N ratio was achieved was used for all following assays. The decreasing trend following the point of maximum S/N seen in Figure S7 for both SLPs (less prominent in the FSLP) occurs due to the accumulation of background noise, a byproduct of incomplete quenching in the closed state which diminishes the sensitivity of the response. The trend presents itself differently in the BSLP because there is no background noise from an external excitation source, therefore the S/N is much higher, especially at lower concentrations.

# 6) MicroRNA detection in a human serum matrix

# Normal mouse serum spiked with miRNA target miR-21: TAGCTTATCAGACTGATGTTGA

was used to prepare a calibration curve. All serum samples were diluted to 25% initial concentration with 100 mM sodium phosphate, 1 mM EDTA, pH 7.4 buffer and boiled for 5 min. Once cooled, 50  $\mu$ L of the serum was added to each well before the addition of synthetic target or BSLP. The total well volume before coelenterazine injection

The mismatch probes contained the following mutations to the wild-type miR21 sequence: single mismatch (SM) - T6A; double mismatch (DM) - A12T, G18C; triple mismatch (TM) - T5A, A12T, G18C.

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was still held at 100 µL.

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*Figure S6.* 10 pmol of target was used to characterize the FSLP, and 4 pmol for the BSLP. These points were chosen because they both fell in the middle of the initial calibrations run according to previous methods.<sup>2</sup> (**A**) The fluorescent emission of varying amounts of FSLP in the presence of 10 pmol of target. (**B**) The background normalized S/N response of varying amounts of FSLP in the presence of 10 pmol of target.



*Figure S7.* To determine the optimal concentration of SLP to use in the assay design, the amount of SLP was varied while holding the concentration of target constant.