SUPPORTING INFORMATION

Enzymatic Beacons for Specific Sensing of Dilute Nucleic Acid and Potential Utility for SARS-CoV-2 Detection

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**Supporting Figure 1.** E-beacon (Eb.2') prepared with solid phase synthesized monosterylated oligo displays favorable signal to noise ratio and limited data scatter. Bioluminescence from samples with Eb.2’ was measured after 10-minute incubation with complementary oligonucleotide (Signal, green, n=48) or with noncomplementary oligonucleotide (Noise, red, n=48). The E-beacon was present at $8 \times 10^{-11}$ M final; oligonucleotide at $1 \times 10^{-7}$ M; temperature, 25 °C; substrate, furimazine.

**Supporting Figure 2.** S/N ratio exceeds 5-fold with sub-picomolar Eb.19 (WT). Target oligonucleotides was held constant at $1 \times 10^{-7}$ M while the Eb.19 (WT) was titrated from $1 \times 10^{-8}$ M to $1 \times 10^{-16}$ M. Signal to noise ratios are plotted on the Y axis.
Supporting Figure 3. Bioluminescence of Eb.19 (E484K) with increasing target oligo concentration after 10 min or 3 hr incubation. Data were fit to a hyperbolic binding isotherm to calculate EC$_{50}$ values. The EC$_{50}$ was 2.54×10$^{-9}$ M for 10 min incubation; for 3 hr, EC$_{50}$ was 0.11×10$^{-9}$ M. Eb.19 (E484K) was present at 8×10$^{-11}$ M.

Supporting Figure 4. Unquenching of Eb.2' by digestion of the hairpin component of Eb.2' with DNase-1 produced similar S/N ratios compared to hybridization driven unquenching. Eb.2' (1×10$^{-8}$ M, final) was mixed with DNase 1 (6 units) and DNase 1 buffer (from New England Biolabs) at 25 °C and analyzed for bioluminescence at selected intervals. The S/N was calculated from the ratio of bioluminescence in samples +/- DNase 1. The incubation period varies from 0 min to 90 min.
1. Materials:

Chemical
All chemicals were obtained from commercial suppliers and used directly unless otherwise mentioned: Fos-Choline-12 (Anatrace); Imidazole (Acros Organics); Triethylammonium acetate (Calbiochem); Dimethyl sulfoxide (EMD Millipore Corp.); Acetonitrile, glycerol tris(2-carboxyethyl)phosphate, Tris Base (Fisher Scientific Inc.); MeOH (Macron Fine Chemicals); Ampicillin, Isopropyl β-d-1-thiogalactopyranoside (MP Biomedicals); Nano-Glo® Luciferase Assay System (Promega); N-(3-Dimethylaminopropyl)-N’-ethylenediisocyanate hydrochloride, n-butanol, LB agar (miller), Luria Bertani Broth (Sigma); 23, 24-BISNOR-5-CHOLENIC ACID-3β-OL (Steraloids); KCl, MgCl₂ (VWR). The CPG solid supports (3’-dabcyl-CPG or dT-CPG, 1.0 µM), DNA phosphoramidites (dT-CE, dABZ-CE, dCAc-CE and dGdmf-CE), 5’-carboxy modifier-C10 amidite, 0.25 M tetrazole in acetonitrile, capping and oxidation solutions(Glen Research).

Plates
Corning® 96 Well Black Polystyrene Microplate (#3650)
Dialysis chambers: EMD Millipore Corp. D-Tube™ Dialyzer Maxi, MWCO 12-14 kDa (#71510)
Concentrators: Corning® Spin-X® UF 6 mL Centrifugal Concentrator, 5,000 MWCO Membrane. (#431482)

Buffers
Bacterial Cell Lysis buffer: 0.5 % Triton X-100, 0.05 M K₂HPO₄, 0.4 M NaCl, 0.1 M KCl, 10 % glycerol, 0.01 M imidazole, pH=7.3.
Ni-NTA Bind buffer: 1 M NaCl, 0.04 M Na₂HPO₄, 0.06 M imidazole, 20 % glycerol, pH=7.5.
Ni-NTA Elution buffer: 0.02 M Na₂HPO₄, 0.5 M NaCl, 0.5 M imidazole, 10% glycerol, pH=7.3.
Agarose gel extraction buffer: 20mM Tris HCl, pH=7.4.
Luciferase Assay DNA hybridization buffer: 100 mM KCl, 1mM MgCl₂, 10 mM Tris HCl, pH=8.0.
2. Methods:

2A. Protein expression/ purification

Two C-terminal His-tagged Nluc-HhC precursor constructs were used in this study. The first construct Nluc-HhC has been described previously 1. The second construct, Nluc-HhC(D46H)-SUMO, differs in using a gain-of-function HhC mutant 2 along with a SUMO tag for enhanced expression and solubility. E. coli BL21(DE3) containing expression plasmid for each His-tagged Nluc-HhC precursor was grown at 37 °C in 50 ml of LB broth with carbenicillin (100 µg/ml) and 250 RPM shaking. Once OD_{600} reached 0.6-0.8, IPTG was added to the culture (0.5 mM, final) to induce expression. After 18-20 hours at 16°C, bacterial cells were harvested by centrifugation at 10,000 RPM for 10 mins and the pellet was resuspended in bacterial lysis buffer (3 ml). After 3 freeze (-80 °C) / thaw cycles and sonication, insoluble material was removed by centrifugation at 10,000 RPM for 60 min. To the clarified lysate, an equal volume of ice chilled 2x Ni-NTA bind buffer was added. The solution was applied to Ni-NTA spin columns (GE Health). His-tagged precursor was purified according to the manufacture’s protocol. Purified Nluc-HhC or Nluc-HhC(D46H)-SUMO precursor protein was stored at -80 °C in Ni-NTA elution buffer with added TCEP (5× 10⁻³ M).

Supporting Figure 5. SDS-PAGE images showing the results of Nluc-HhC precursor protein purification. (A) Nluc-HhC precursor protein purification. (I) Nluc-HhC (44kDa). (B) Nluc-D46H-SUMO precursor protein purification. (II) Nluc-HhC(D46H)-SUMO (58kDa), (III) D46H-SUMO (39kDa). The presence of (III) is caused by spontaneous self-cleavage (hydrolysis) of the autoprocessing domain, HhC(D46H)-SUMO, fragment from Nluc.
2B. Solution-based EDC oligonucleotide sterylation (used for EB.1-3)

To 200 µl of DMSO, we first added 4.33 mg (12.5 µmol) of 23, 24-bisnor-5-cholenic acid-3β-ol (1) and 23.95 mg (125 µmol) of N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC). After incubation at room temperature for 30 min, 1.5 mg (12.5 µmol) of 4-Dimethylaminopyridine (DMAP) was added and incubated at room temperature for another 5 min. Last, 50 µl of 100 µM 5’-amino modified oligo (2) (in H₂O) was added and the solution was vortexed gently at room temperature overnight. The coupling reaction was extracted by adding 20 µl of 3 M sodium acetate, pH 5.2, and 1 ml of n-butanol. After a brief vortex and incubation at -80 °C for 1 hour, oligonucleotide was collected as a precipitate by centrifugation at 14,000 RPM for 20 min. The pellet was resuspended in 50 µl of water. Sterylated oligonucleotide (steramer) (3) was separated from sterol-free oligonucleotide by RP-HPLC, see below.

Supporting Figure 6. Reversed phase HPLC chromatogram of the sterol modified oligonucleotide with 3’ quencher prepared by EDC-based sterylation. Sterol modified oligonucleotides (starred).
2C. Synthesis and purification of sterol amine (5) for solid phase oligo coupling (for EB.19s)

To 6 ml of anhydrous DMF, 173.5 mg (0.5 mmol) of 23, 24-bisnor-5-cholenic acid-3β-ol (1), 435 ul (2.5 mmol) of N,N-Diisopropylethylamine (DIPEA), and 165.6 mg (0.55 mmol) of N,N,N',N'-Tetramethyl-O- (N-succinimidyl) uronium tetrafluoroborate (TSTU) were added. The mixture was stirred at room temperature for 30 minutes. After that the mixture was added to 2 ml of 0.75 M 1,7-Diaminoheptane (4) in anhydrous DMF dropwise. Then the final reaction mixture was stirred at room temperature for 20 hours. After that the reaction mixture was washed with dichloromethane (10 ml) and saturated NaHCO₃ solution (10 ml) for three times, dried over Na₂SO₄, and evaporated under N₂ flow to afford 167.7 mg crude sterol amine (5) as a white to yellow solid.

The crude sterol amine (5) was dissolved in 1 ml of methanol and purified over a Restek viva C18 5 µm HPLC column (250×4.6 mm) using a gradient elution from 0% acetonitrile to 100% acetonitrile over 25 minutes. The flow rate was 1 mL/min and eluate was monitored at 210 nm. Sample injection volume was 100 µl. After HPLC purification, the solvent was evaporated to afford sterol amine (5) (144.6 mg, 63%) as a white solid.

**NMR Characterization of (5):**

¹H NMR spectra were acquired with Bruker Avance III HD 400 (400MHz) spectrometer at 25 °C. CD₃OD (Sigma) was used as NMR solvent. ¹H chemical shifts are reported as δ in units of parts per million (ppm) relative to methanol-d (3.31, s)

1H NMR (400MHz, CD₃OD) δ 5.34 (dd, 1H), 3.40 (m, 1H), 3.16 (m, 1H), 3.11 (m, 1H), 2.91 (t, 2H), 2.17 (dd, 1H), 1.38 (s, 6H), 1.14 (d, 2H), 1.03 (s, 3H), 0.75 (s, 3H). ¹³C NMR (100MHz, CD₃OD) δ 178.21, 140.86, 120.93, 71.00, 56.47, 52.61, 50.30, 43.64, 42.02, 41.60, 39.60, 39.30, 38.53, 37.14, 36.29, 31.88, 31.58, 30.89, 28.87, 28.34, 27.13, 26.98, 26.27, 25.96, 23.94, 20.76, 18.48, 16.56, 11.09.
**Supporting Figure 7.** $^1$H NMR of the synthetic sterol amine (5).

**Supporting Figure 8.** $^{13}$C NMR of the synthetic sterol amine (5)
Supporting Figure 9. LC-MS analysis of the synthetic sterol amine (5).

2D. Solid Phase Steramer Synthesis

The sterylated-DNA oligomers (steramers, Table 1) were synthesized on a 1.0 µM scale (3’-dabcyl-CPG or dT-CPG) using standard DNA synthesis protocols on an automated Expedite 8909 DNA/RNA synthesizer. After synthesizing the desired DNA sequence, the 5’-end was modified with 5’-carboxy modifier-C10 carrying a reactive NHS ester. The final detritylation step was eliminated after coupling with the 5’-carboxy modifier-C10. Prior to the next coupling with sterol amine, the solid support was washed with acetonitrile followed by drying with nitrogen purging for 5 minutes.

The manual coupling of sterol amine (5) (10 eq.) with the DNA-NHS ester on the CPG solid support was performed in anhydrous DMSO containing 10% DIPEA at room temperature for 2 hours using the push-pull syringe method. After the coupling, the column was thoroughly washed with 5×1 mL of acetonitrile and dried. The steramers (7) were cleaved from the solid support using 40% methyl amine in water (2×0.8 mL) at room temperature for 2 hours. The cleavage solution was lyophilized to get the crude steramers. The exposure to light was avoided as much as possible while handling.
the 3’-dabcyl labeled steramers synthesis. The identity of all the synthesized crude steramers were confirmed by the MALDI-ToF mass analysis (Table 1). The crude samples of all the steramers showed ~85-90% purity by RP-HPLC.

Supporting Table 1: List of steramers prepared by solid phase synthesis

| E-beacons       | *Sequence (5’-3’) | Mass calcd./found |
|-----------------|-------------------|-------------------|
| Eb.2’           | sterol- CGCTC CCAAAAAAAAAACC GAGCG- dabcyl | 8780/8783         |
| Eb.19 (E484)    | sterol- CGCTC TGGTGTTGAAGGTTT GAGCG- dabcyl | 8909/8912         |
| Eb.19 (E484K)   | sterol- CGCTC TGGTGTTAAAGGTTT GAGCG- dabcyl | 8893/8898         |

* The underlined regions self-anneal to form the hairpin stem. The nucleotides in black color were for the target RNA/DNA detection. Sterol moiety at the 5’-end was introduced for the bioconjugation with nanoluciferase and the dabcyl unit at 3’-end was as a dark fluorescence quencher.

Supporting Figure 10. Reversed phase HPLC chromatogram of the sterol modified oligonucleotide with 3’ quencher prepared by solid phase.
2E. General Conditions for E-beacon bioconjugation by HhC

To prepare Nluc-hairpin nucleic acid conjugates, Nluc-HhC precursor protein (2×10^6 M, final), Fos-choline 12 (1.5×10^3 M, final), Tris(2-carboxyethyl) phosphine hydrochloride (TCEP, 5×10^3 M, final), Bis-Tris buffer (0.05 M, final, pH 7.1), ethylenediaminetetraacetic acid (EDTA, 5×10^4 M, final), NaCl (0.1 M, final) were mixed. To that solution, Steramer (1×10^4 M, final) was added and the reaction was incubated at 16 °C overnight.

2F. E-beacon isolation by agarose gel extraction

Agarose gel extraction was used as a rapid means of isolating E-beacon. E-beacon conjugation reaction (100 µl) was combined with 6X gel loading buffer (20 µl) and separated on 2% agarose gel containing GelRed® Nucleic Acid Gel Stain (followed the manufacturer’s “precast protocol”). The gel was run at 90 V in 1 x TAE buffer until the sample loading dye front was approximately 95% toward the end of the gel. E-beacon conjugate was visualized with BioRad Gel Doc® (UV tray), then excised, diced, transferred to an Eppendorf tube and soaked in Tris buffer (3 ml, 20 mM pH7.4) at 4 °C overnight. After centrifugation to gel fragment, the supernatant was concentrated by a Spin-X® UF Concentrator (Corning), 5 kDa MWCO.

Supporting Figure 11. Image of the agarose gel for E-beacon purification. Nucleic acid moieties in the agarose gel were visualized by GelRed® nucleic acid gel stain giving bright bands.
2G. E-beacon Bioluminescence Measurements

To perform luminescence reading, 25 µl of E-Beacon solution was combined with 25 µl of sample nucleic acid in the DNA hybridization buffer a Corning® 96 Well Black Polystyrene Microplate. After incubation at 25 °C for the selected interval, we added 25 µl of NanoGlo® Luciferase Assay System substrate (Promega). After another 5 min, bioluminescence was measured using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek).

References

1. Zhang, X.; Xu, Z.; Moumin, D. S.; Ciulla, D. A.; Owen, T. S.; Mancusi, R. A.; Giner, J. L.; Wang, C.; Callahan, B. P., Protein-Nucleic Acid Conjugation with Sterol Linkers Using Hedgehog Autoprocessing. Bioconjug Chem 2019, 30 (11), 2799-2804.

2. Zhao, J.; Ciulla, D. A.; Xie, J.; Wagner, A. G.; Castillo, D. A.; Zwarycz, A. S.; Lin, Z.; Beadle, S.; Giner, J. L.; Li, Z.; Li, H.; Banavali, N.; Callahan, B. P.; Wang, C., General Base Swap Preserves Activity and Expands Substrate Tolerance in Hedgehog Autoprocessing. J Am Chem Soc 2019, 141 (46), 18380-18384.