Supplemental Information for

Fluorogenic Sensor Platform for the Histone Code Using Receptors from Dynamic Combinatorial Libraries

Brendan C. Peacor, Christopher M. Ramsay, and Marcey L. Waters*

Peptide Synthesis

All peptide synthesis was performed on a Tetras Peptide Synthesizer using CLEAR-Amide resin from Peptides International using Fmoc N-terminal protected amino acids with protected side chain functionality. Coupling reagents were HOBt/HBTU in DMF with 8 equivalents of DIPEA. After synthesis, all peptides were acylated using 5% acetic anhydride and 6% 2,6-lutidine in DMF, followed by cleavage and global deprotection using 95% TFA, 2.5% TIPS, and 2.5% H_2O for four hours. Trimethylated peptides were synthesized by adding 2 equivalents of Fmoc-Lys$(Me)_2$-OH. Following N-terminal acylation, but prior to cleavage, the dimethyl lysine was further methylated using ten equivalents of MTBD and MeI. Peptides were purified using semi-preparative reverse phase HPLC using a XBridge Peptide C18 column with a linear gradient of A and B (Solvent A: 95% H_2O/5% CH_3CN with 0.1% TFA; Solvent B: 95% CH_3CN/5% H_2O with 0.1% TFA) and monitored at 214nm. Peptides were then lyophilized and characterized by ESI-MS.

Receptor Synthesis

All of the receptors used in the study were synthesized and purified following previously published protocols and lyophilized to powder for storage at -20°C prior to use.[1–4]

Receptor Fluorescence Titration

Fluorescence quenching experiments were performed using purified receptors and commercially purchased Lucigenin. The titration for **A_2B**, **A_2D**, and **A_2N** used 5 µM of fluorophore and increasing concentrations of receptor 25 mM K_2HPO_4, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, and 0.01% Triton X-100, while **A_2G** was performed in a 50 mM Glycine buffer, pH 9.15, using 1 µM of fluorophore. Plates were centrifuged and incubated for 15 minutes prior to reading on a POLARStar Omega (BMG Labtech) using excitation: 370 nm, emission: 510 nm. Binding curves were fit using a 1:1 fluorescence quenching curve shown below.

$$I = \frac{I_0 + I_\infty \left(\frac{[L]}{K_d}\right)}{1 + \left(\frac{[L]}{K_d}\right)}$$

Where I is the observed fluorescence intensity, I_o is the initial fluorescence, I_∞ is the fluorescence at binding saturation, [L] is the concentration of added receptor, and K_d is the dissociation constant.

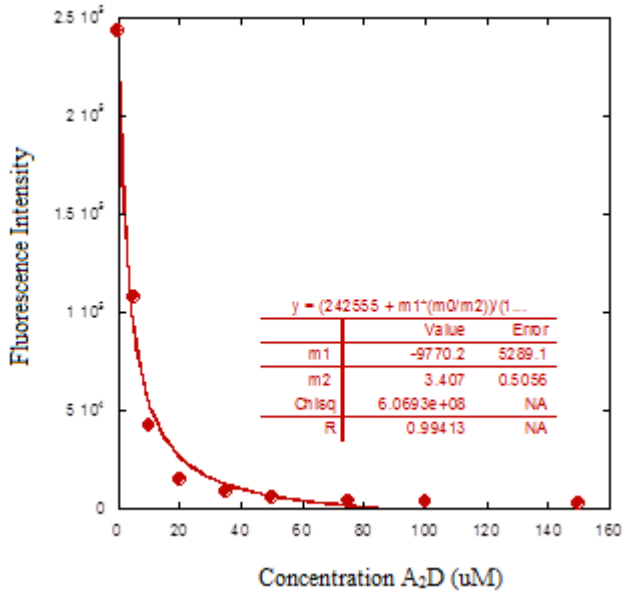

Figure S1. Fluorescence quenching of **LCG** (5μM) caused by binding to **A_2D** with a K_d of 3.4 ± 0.5 μM.

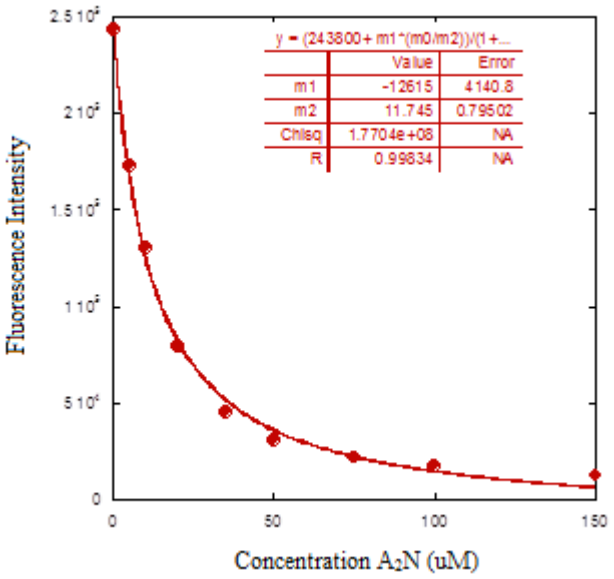

Figure S2. Fluorescence quenching of **LCG** (5μM) caused by binding to **A_2N** with a K_d of 11.7 ± 0.8 μM.

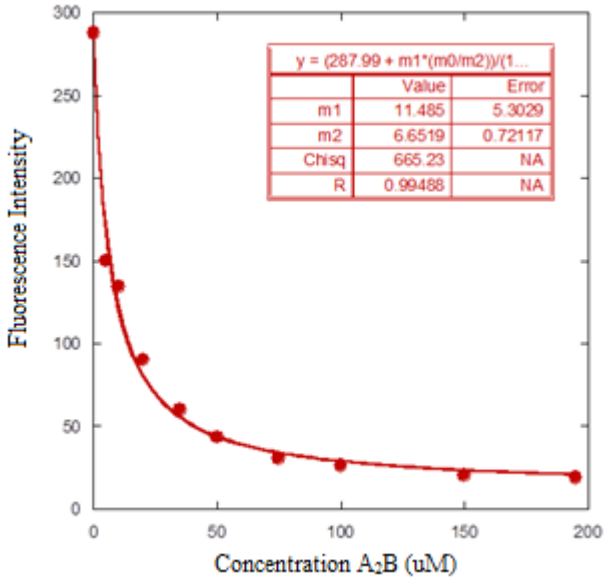

Figure S3. Fluorescence quenching of **LCG** (5μM) caused by binding to **A_2B** with a K_d of 6.7 ± 0.7 μM.

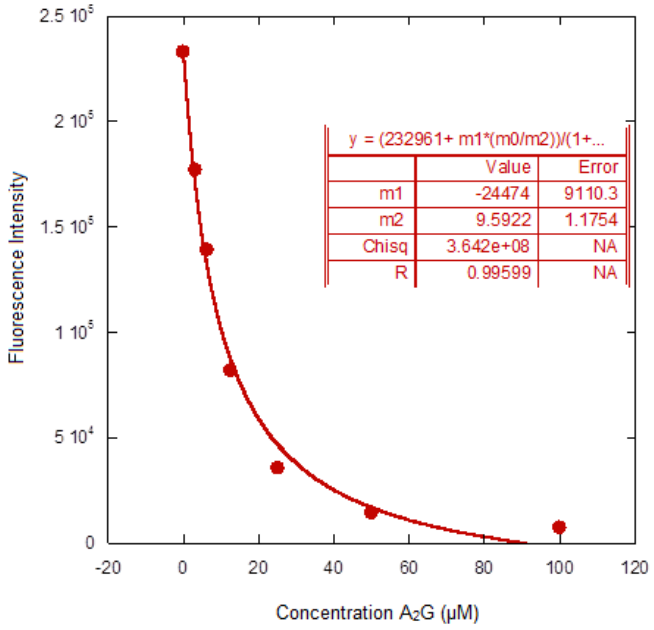

Figure S4. Fluorescence quenching of **LCG** (1μM) caused by binding to **A_2G** with a K_d of 10 ± 1 μM.

Indicator Displacement Assay (IDA) Development

Fluorescence titrations were performed using purified **A_2N** and **LCG** in fixed concentrations as well as purified H3 1-12 peptides with either no modification or dimethyl lysine 9 in 50 mM Glycine buffer, pH 9.15. In a 96 well plate, each reagent combination shown in figure **S5** was performed in triplicate, with centrifugation followed by equilibration of the plate for 15 minutes prior to reading using previously described settings.

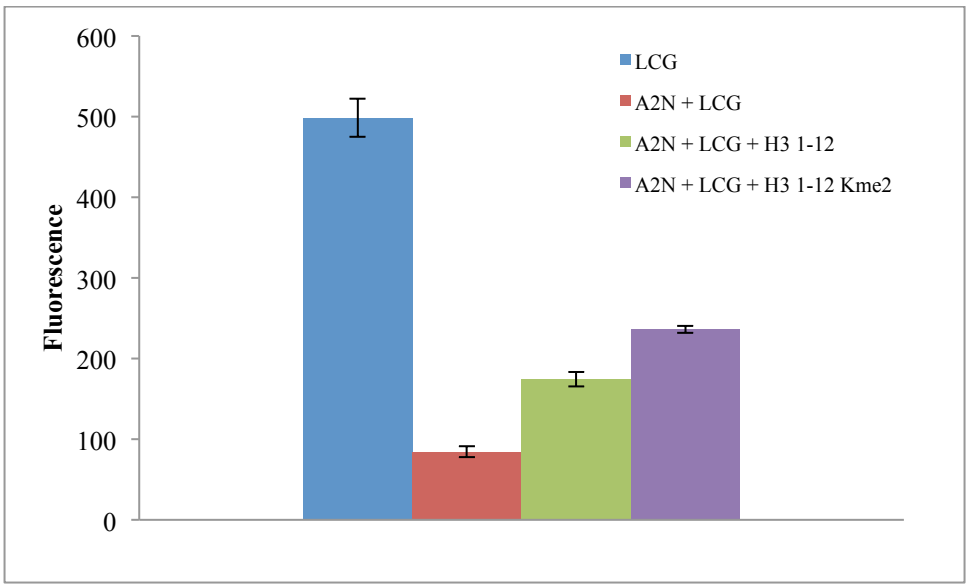

Figure S5. Fluorescence of LCG (2.5 μM) followed by addition of A_2N (25 μM), quenching signal. Addition of unmethylated H3 1-12 peptide (15 μM) recovered fluorescence, though more signal was observed in the presence of the higher affinity binder H3 1-12 Kme2.

384-well Sensor Array

The 384 well sensor array utilized four sensors, **A_2B** (10 μM), **A_2D** (5 μM), **A_2G** (10 μM) and **A_2N** (15 μM), with concentrations determined using previously determined extinction coefficients on a Nanodrop UV-Vis. [1–4] Each sensor was paired with **LCG** (1 μM). The assay was run at 9 μL total volume per well with twenty replicates per analyte. Each well contained 3 μL of each peptide (final concentration 15 μM), **receptor**, and **LCG** for a total volume of 9 μL per well. The plates were centrifuged for 1 minute and incubated for 15 minutes, followed by reading on a POLARStar Omega (BMG Labtech) using excitation: 370 nm, emission: 510 nm. Each analyte was run using a single plate, with control wells containing LCG alone and receptor/LCG to monitor the maximum fluorescence signal as well as the quenched state of the

system. Fluorescence signal was normalized prior to analysis by dividing the fluorescence response by the fluorescence of **LCG** alone.

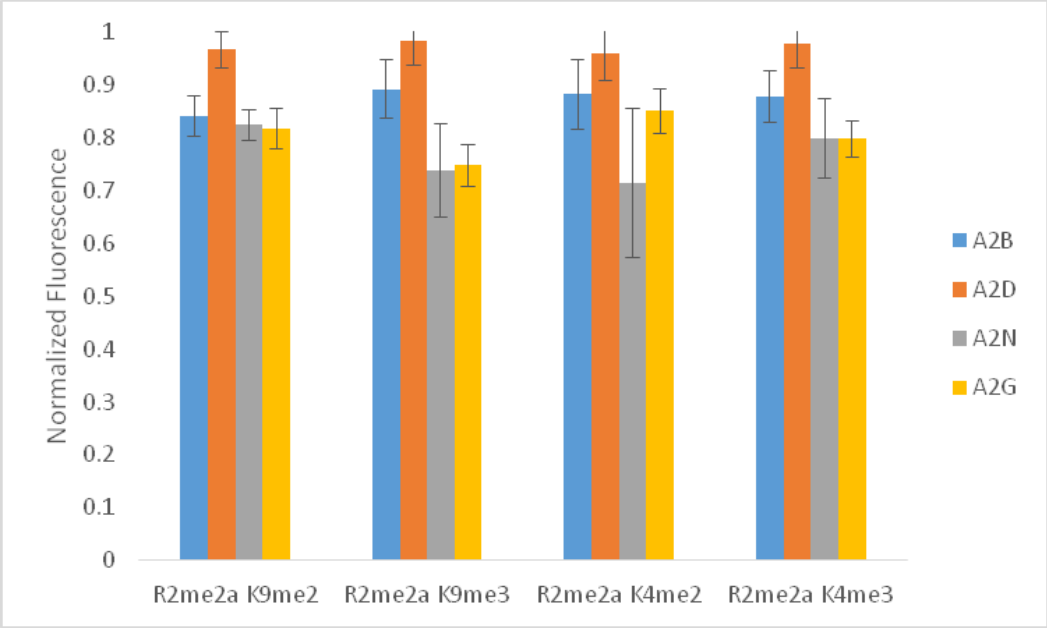

Figure S6. Fluorescence titration of **LCG•Receptor** sensors with peptide analytes bearing multiple methylations in the sequence Ac-AR(Me)$_n$TK(Me)$_n$QTARK(Me)$_n$STGY-NH$_2$ (15 μM). Each sensor utilized a fixed concentration of LCG (1 μM) and receptor (A_2B - 10 μM; A_2D – 5 μM; A_2N – 15 μM; A_2G – 10 μM). Each sensor array was run in 50 mM Glycine buffer, pH 9.15 at room temperature in a 384 well plate. Fluorescence was normalized prior to plotting (F_n= F/F_∞). Error bars are plotted using twenty replicates for each analyte.

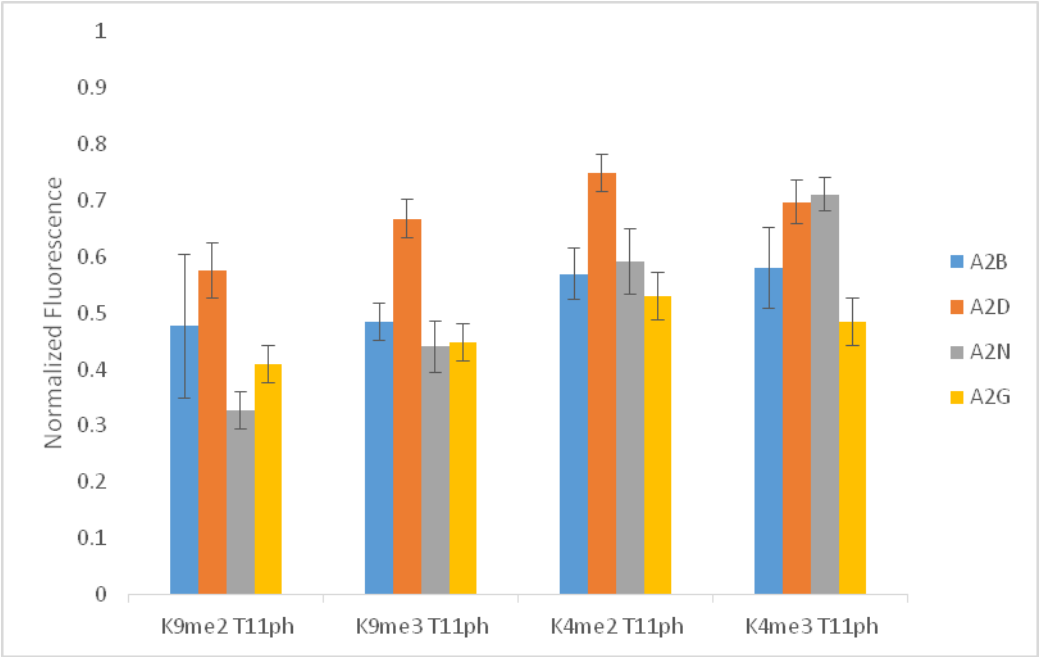

Figure S7. Fluorescence titration of **LCG•Receptor** sensors with peptide analytes bearing methylation and phosphorylation in the sequence Ac-ARTK(Me)$_n$QTARK(Me)$_n$ST(Phos)$_n$GY-NH_2 (15 μM). Each sensor utilized a fixed concentration of LCG (1 μM) and receptor (A_2B - 10 μM; A_2D – 5 μM; A_2N – 15 μM; A_2G – 10 μM). Each sensor array was run in 50 mM Glycine buffer, pH 9.15 at room temperature in a 384 well plate. Fluorescence was normalized prior to plotting ($F_n = F/F_\infty$). Error bars are plotted using twenty replicates for each analyte.

Linear Discriminate Analysis

LDA was performed using SysStat13. Prior to analysis, each analyte was normalized to the maximum fluorescence of the plates control well of **LCG** alone (F/F_∞). Additionally, each series of replicates was sorted from smallest response to largest response for each sensor, to decouple experimental error bias from the statistical analysis. The discriminate analysis was performed with all groups equal at 0.001 tolerance, and the resulting factors were plotted. The jackknife analysis was performed automatically in the software by classifying the data set while leaving one replicate at a time out, then re-submitting said replicate as a blind point to if the classification was upheld.

Table S1. Classification matrix for peptides bearing single PTMs. Analyte labels on the left were input into the system, columns represent system classification.

	H3	K9me3	K4ac	R2me2a	T11ph	%correct
H3	20	0	0	0	0	100
K9me3	0	20	0	0	0	100
K4ac	0	0	20	0	0	100
R2me2a	0	0	0	20	0	100
T11ph	0	0	0	0	20	100
Total	20	20	20	20	20	100

Table S2. Jackknife **c**lassification matrix for peptides bearing single PTMs. Analyte labels on the left were input into the system, columns represent classification of the analyte left out of initial data set analysis.

	H3	Kme3	K4ac	R2me2a	T11ph	%correct
H3	20	0	0	0	0	100
K9me3	0	20	0	0	0	100
K4ac	0	0	20	0	0	100
R2me2a	0	0	0	20	0	100
T11ph	0	0	0	0	20	100
Total	20	20	20	20	20	100

Table S3. Classification matrix for peptides bearing multiple sites of methylation. Analyte labels on the left were input into the system, columns represent system classification.

	R2me2a K9me2	R2me2a K9me3	R2me2a K4me2	R2me2a K4me3	%correct
R2me2a K9me2	20	0	0	0	100
R2me2a K9me3	0	20	0	0	100

R2me2a K4me2	1	0	19	0	95
R2me2a K4me3	0	0	0	20	100
Total	21	20	19	20	99

Table S4. Jackknife **c**lassification matrix for peptides bearing multiple sites of methylation. Analyte labels on the left were input into the system, columns represent classification of the analyte left out of initial data set analysis.

	R2me2a K9me2	R2me2a K9me3	R2me2a K4me2	R2me2a K4me3	%correct
R2me2a K9me2	19	0	1	0	95
R2me2a K9me3	0	20	0	0	100
R2me2a K4me2	1	0	19	0	95
R2me2a K4me3	0	0	0	20	100
Total	20	20	20	20	98

Table S5. Classification matrix for peptides bearing methylation and phosphorylation. Analyte labels on the left were input into the system, columns represent system classification.

	T11ph K9me2	T11ph K9me3	T11ph K4me2	T11ph K4me3	%correct
T11ph K9me2	20	0	0	0	100
T11ph K9me3	0	20	0	0	100
T11ph K4me2	0	0	20	0	100
T11ph K4me3	0	0	0	20	100
Total	20	20	20	20	100

Table S6. Jackknife **c**lassification matrix for peptides bearing methylation and phosphorylation. Analyte labels on the left were input into the system, columns represent classification of the analyte left out of initial data set analysis.

	T11ph K9me2	T11ph K9me3	T11ph K4me2	T11ph K4me3	%correct
T11ph K9me2	20	0	0	0	100
T11ph K9me3	0	20	0	0	100
T11ph K4me2	0	0	20	0	100
T11ph K4me3	0	0	0	20	100
Total	20	20	20	20	100