Deep Quench: An Expanded Dynamic Range for Protein Kinase Sensors

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Supporting Information

Experimental section

General reagents and solvents were purchased from Fisher or Aldrich. CLEAR Rink amide resin and Fmoc-2,6-dioxoaminooctanoic acid, HCTU [1H-benzotriazolium 1-[*bis*(dimethylamino)methylene]-5-chloro-,hexafluorophosphate (1-),3-oxide], and HOBt-Cl (6-chloro-1-hydroxy-1H-benzotriazole) purchased from Peptides International (Louisville, KY). Fmoc-ß-Ala-OH, Fmoc-aminobutyric acid, Fmoc-aminovaleric acid, Fmoc-aminohexanoic acid, and Fmoc-aminooctanoic were purchased from Advanced Chem Tech (Louisville, KY). Fmoc-Dap(Mtt)-OH was purchased from Novabiochem (La Jolla, CA). PKA murine catalytic subunit plasmid and the GST-14-3-3τ plasmid were generous gifts from Dr. Susan Taylor and Dr. Alistair Aitken, respectively.

Synthesis of Peptide Libraries. Peptides were synthesized by standard solid phase synthesis using Fmoc chemistry. The Fmoc protecting group was removed with 20% piperidine in dimethylformamide (DMF) (1x5 min, 1x20 min). Sequential coupling of Fmoc protected amino acids was achieved with 3 equiv. Fmoc amino acid, 3 equiv. HCTU, 3 equiv. HOBt-Cl, and 6 equiv. diisopropylethylamine (DIPEA). Completion of each reaction was monitored with the Kaiser and chloranil tests. Resins were washed between steps with DMF, isopropyl alcohol (IPA), and DCM. For peptides **P1** - **P5**, the free N-terminal Gly^1 was acylated with 20 equiv. of acetic anhydride in dissolved in 1:1 pyridine:DMF. The 4-methyltrityl protecting group on Dap(Mtt) was orthogonally removed using 5% trifluoroacetic acid (TFA) and 5% triisopropylsilane (TIPS) in DCM (5 min incubation). The resulting free ß-amine was acylated with 3 equiv. 1-pyreneacetic acid in DMF containing 3 equiv. HCTU, 3 equiv. HOBt-Cl, and 6 equiv. of DIPEA. The free N-termini of peptides **P6** - **P11** were directly acylated with 1-pyreneacetic acid following the Fmoc deprotection of terminal ß-alanine (ßAla), aminobutyric acid (Abu), aminovaleric acid (Ava), aminohexanoic acid (Ahx), aminooctanoic acid (Aoc), and amino-3,6-dioxoaminooctanoic acid (miniPEG^{TM}) groups, respectively. The remaining orthogonal protecting groups were removed and the peptides cleaved from their resins with 95% TFA, 5% water, 5% TIPS (3 hr). The peptides were isolated via filtration of the resin, precipitation with ice-cold diethyl ether, and centrifugation. The precipitates were air dried and purified by reverse-phase HPLC using a linear gradient (3% - 40% acetonitrile in water with 0.1% TFA over 40 min). The peak corresponding to the

desired peptide was collected, frozen, and lyophilized. The resulting white, flocculent peptides were characterized by electrospray ionization mass spectrometry: **P1** Ac-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Dap(Pyr)-Pro-amide (m/z calculated 1403.72, found 1403.80); **P2** Ac-Gly-Arg-Thr-Gly-Arg-Arg-Dap(Pyr)-Ser-Tyr-Pro-amide (m/z calculated 1419.72, found 1419.60); **P3** Ac-Gly-Arg-Thr-Dap(Pyr)-Arg-Arg-Phe-Ser-Tyr-Pro-amide (m/z calculated 1507.75, found 1509.47); **P4** Ac-Gly-Arg-Dap(Pyr)-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (m/z calculated 1463.72, found 1464.87); **P5** Ac-Dap(Pyr)-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (m/z calculated 1507.75, found 1509.93); **P6** Pyr-ßAla-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (m/z calculated 1507.75, found 1509.47); **P7** Pyr-Abu-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (m/z calculated 1521.76, found 1523.80); **P8** Pyr-Ava-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (m/z calculated 1535.78, found 1537.40); **P9**, Pyr-Ahx-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (m/z calculated 1549.79, found 1551.60); **P10** Pyr-Aoc-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (m/z calculated 1577.83, found 1578.73); **P11** Pyr-miniPEG^{TM}-Gly-Arg-Thr-Gly-Arg-Arg-Phe-Ser-Tyr-Pro-amide (m/z calculated 1582.76, found 1583.73).

Identification of Lead Quencher Dyes. The concentration of peptides **P1** – **P11** was adjusted to 50 µM based on the molar excitation coefficient of 22,000 M^{-1} cm^{-1} at 345 nm. The concentrations of 47 dyes were adjusted to 50 µM by weight. The peptides were screened against the dyes on 96 well plates using an HTS 7000 Bio Assay Reader (Perkin Elmer) with 340 nm excitation filter and 380 nm emission filter, a setting of 100 µs integration time, and 5 flashes. Each well contained 5 µM peptide and 5 µM dye in 50 mM Tris-HCl at pH 7.5. Dyes that resulted in the greatest degree of fluorescence quenching were noted.

Chart S1. Lead quencher dyes of pyrene peptides **P1** – **P11**.

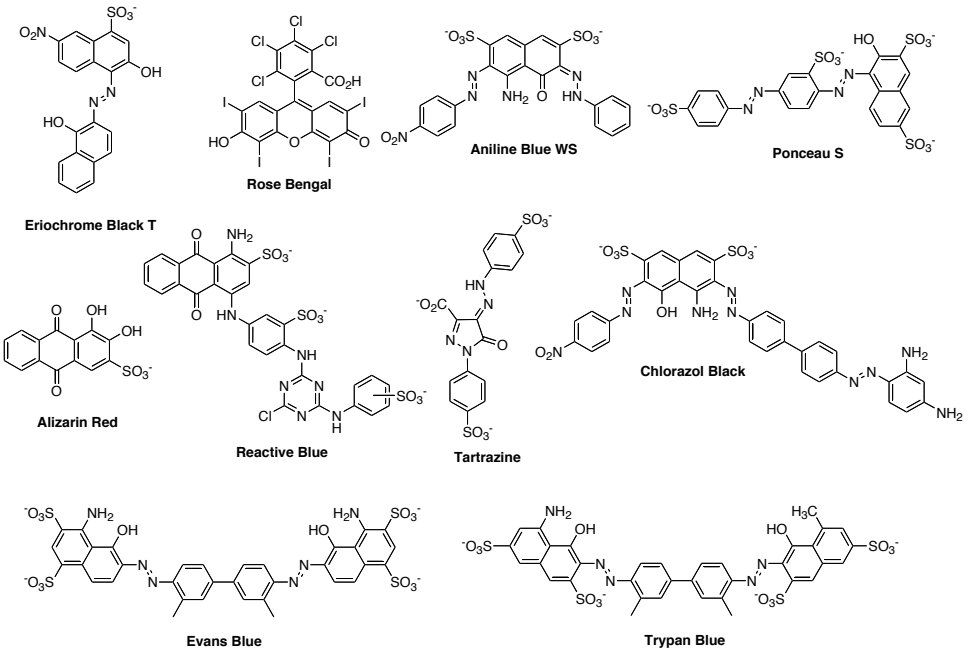


Table S1. Library of Dyes

D1	Acid Green 27
D2	Acid Blue 40
D3	Evans Blue
D4	Acid Alizarin Violet N
D5	Acid Blue 80
D6	Reactive Blue 2
D7	N,N-dimethylnitrosoaniline
D8	Cresol Red
D9	Phenol Red
D10	Methyl Orange
D11	Bromophenol Blue
D12	BUFFER
D13	Xylene Cyanol FF
D14	Disperse Yellow 3
D15	Ethyl Orange
D16	Methylene Blue
D17	Brilliant Blue R
D18	Eriochrome Black T
D19	Alizarin Red
D20	Malachite Green oxalate
D21	Phenolphthalein
D22	Carminic Acid
D23	Nuclear Fast Red
D24	Acid Fuchsin
D25	Acridine Orange
D26	Acridine Yellow G
D27	Aniline Blue WS
D28	Azure A
D29	Azure B bromide
D30	Basic Fuchsin
D31	Bismark Brown Y
D32	Brilliant Yellow
D33	Bromocresol Purple
D34	Chlorazol Black E
D35	Chlorophenol Red
D36	Chrysoidine Y
D37	Erythrosin
D38	Ethyl Violet
D39	Naphthol Blue Black
D40	Methylthymol Blue

D41	Methyl Violet
D42	Ponceau S
D43	Rose Bengal
D44	Rosolic Acid
D45	Safranin O
D46	Serva Violet 49
D47	Tartrazine
D48	Trypan Blue

Acquisition of Apparent K_D Values for Lead Quencher Dyes with Peptide P2. Varying concentrations of 10 dyes, ranging from 0.5 – 500 μM, were added to 5 μM pyrene-labeled **P2** peptide in 100 mM Tris HCl pH 7.5 buffer (96 well plates). A Spectra Max Gemini EM plate reader (Molecular Devices) was used for fluorescence measurements (λ_{ex} = 342 nm and (λ_{em} = 380 nm). Correction for the inner filter effect was made using the antilogarithm of the effective optical density times half the width of the fluorescence well as previously reported (Clin. Chem 23 (12) 2292-2301, 1977). Molar absorbtivities (ε_{342} and ε_{380}) were calculated from single absorbance spectra at a [dye] = 7.81 μM. For all dyes at concentrations below 10 μM, the inner filter effect required a correction of less than 10% in the measured fluorescence. However, at higher concentrations, the effect became significant for strongly absorbing dyes. After correcting for the inner filter effect, the percentage of quench was plotted against the concentration of the dye. A nonlinear regression analysis fit of the data to the rectangular hyperbola model using the Sigma Plot version 8.02 software was used to obtain apparent K_D values.

Table S2. Apparent K_D Values of Lead Quenchers with Peptide P2.

Quencher Dye		**Apparent K_D (µM)**
D3	Evans Blue	2.8 ± 0.8
D6	Reactive Blue	19.6 ± 3.4
D18	Eriochrome Black	14.3 ± 3.3
D19	Alizarin Red	7.3 ± 2.5
D27	Aniline Blue WS	18.1 ± 2.6
D34	Chlorazol Black E	7.7 ± 1.5
D42	Ponceau S	11.2 ± 2.7
D43	Rose Bengal	7.5 ± 1.6
D47	Tartrazine	15.0 ± 2.1
D48	Trypan Blue	11.9 ± 3.6

Acquisition of apparent K_D values for lead quencher/peptide pairs were performed as described above and are reported in the manuscript. In addition, the apparent K_D value for the *phosphorylated* **P5** peptide AcDap(Pyr)RTGRRFS(PO_3^{2-})YP-amide with Rose Bengal is 210 ± 40 nM, slightly tighter than that found for the unphosphorylated AcDap(Pyr)RTGRRFSYP-amide/Rose Bengal pair (400 ± 30 nM).

Figure S1. Percent Fluorescent Quenching of Peptide **P2** As A Function of Concentration of Dyes **1** – **10**.

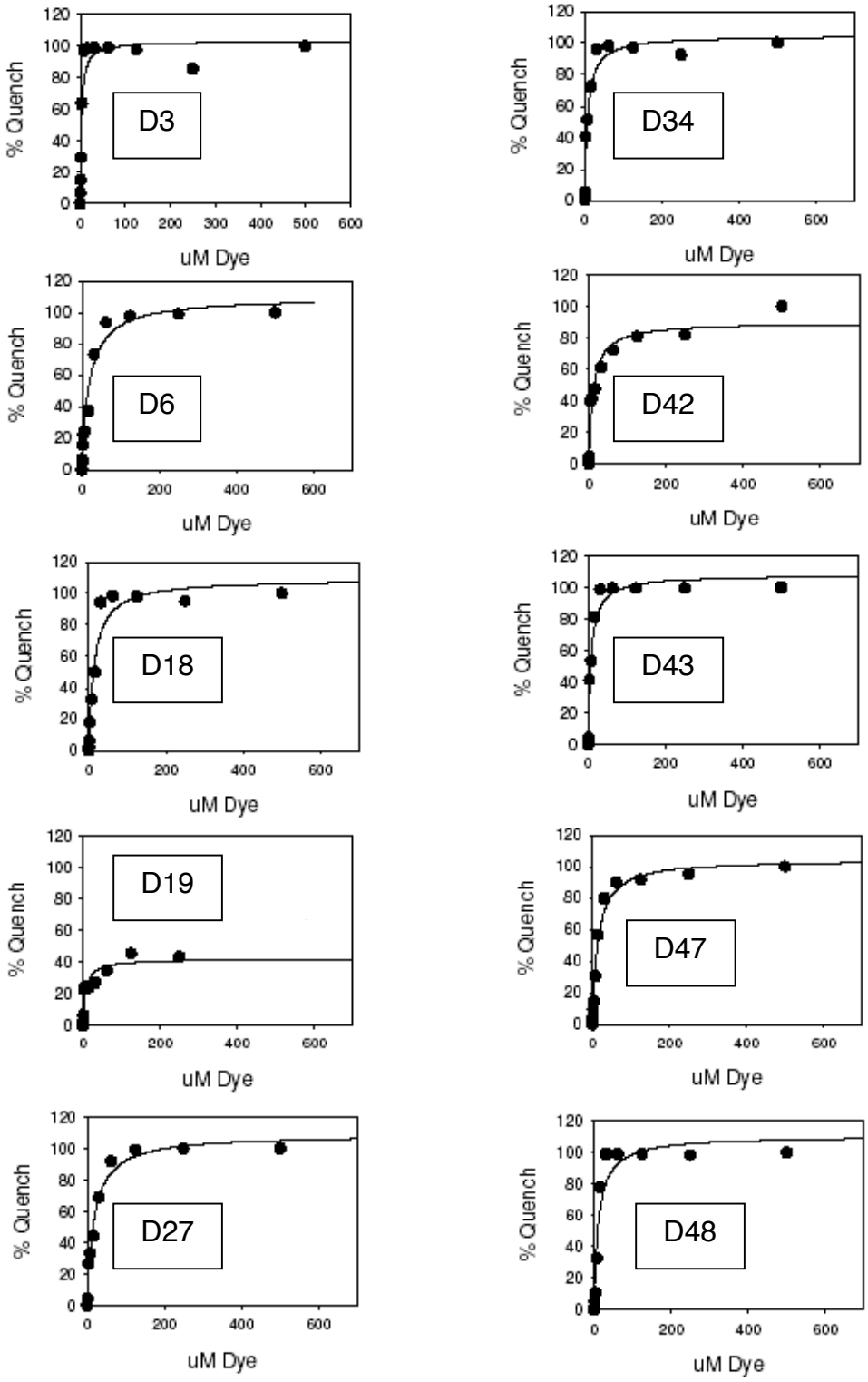
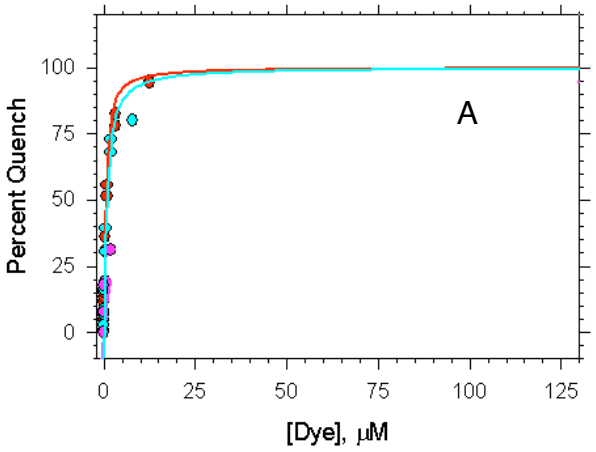
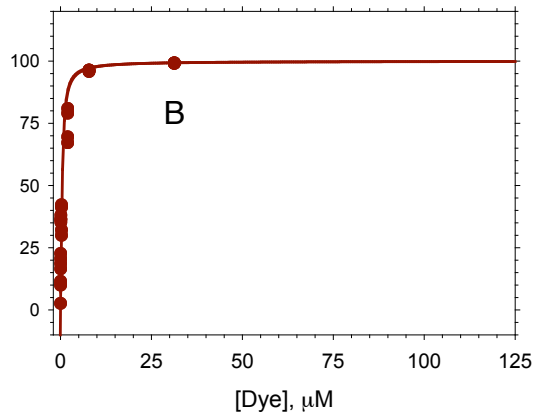


Figure S2. Percent Fluorescent Quenching of pyrene fluorescence in (A) peptide **P5** with Rose Bengal dye (red) and peptide **P9** with Aniline Blue WS dye (cyan), and (B) phosphorylated peptide **P5** and Rose Bengal.





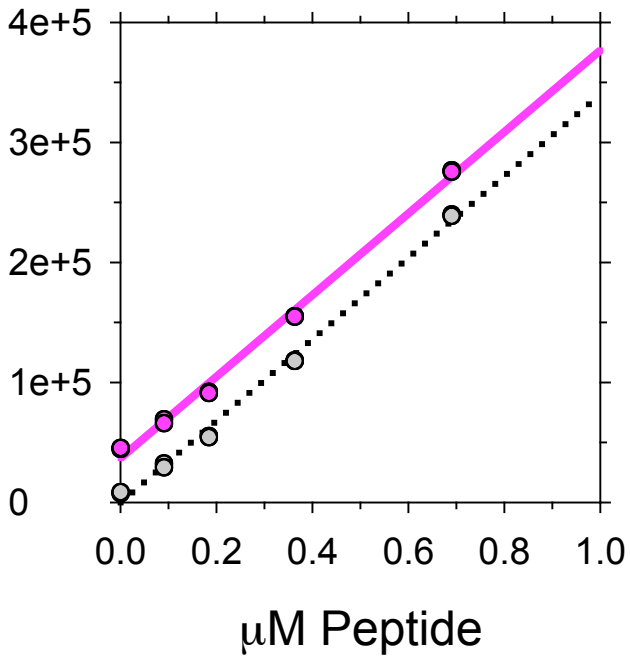
Screening of Lead Quenching Dyes 1 – 10 with Peptides P1 - P11. PKA-catalyzed phosphorylation was initiated by addition of 25 µL of 100 nM PKA enzyme to the following solution: 25 µL 50 µM fluorescent peptide substrates (**P1** – **P11**), 25 µL 20 mM DTT, 25 µL 10 mM ATP, 25 µL 50 mM $MgCl_2$, 25 µL 100 µM 14-3-3τ, 25 µL 0.5 M Tris HCl pH 7.5, 25 µL dye (10 dyes at 4 concentrations, 0.25 mM, 0.5 mM, 1.25 mM 2.5 mM and no dye as a control) to give final volume of 250 µL. The concentrations per well were: 10 nM PKA, 5 µM peptide, 10 µM 14-3-3τ, 1 mM ATP, 5 mM $MgCl_2$, 2 mM DTT, and 25 µM, 50 µM, 250 µM or 500 µM each of 10 different lead dyes in 50 mM Tris at pH 7.5 buffer. The HTS 7000 Bio Assay Reader was set in kinetic mode to monitor the progress of reaction (340 nm excitation filter, 380 nm emission filter, 100 µs and 5 flashes).

Table S3. Control Experiment: Phosphorylation-induced Change in Fluorescence of Pyrene-labeled peptides **P1** - **P11** in the Absence of Quenching Dye.

Pyrene-labeled Peptide		**% Fluorescence Enhancement**
P1	Ac-GRTGRRFSDap(Pyr)P-amide	0
P2	Ac-GRTGRRDap(Pyr)SYP-amide	51%
P3	Ac-GRTDap(Pyr)RRFSYP-amide	19%
P4	Ac-GRDap(Pyr)GRRFSYP-amide	40%
P5	Ac-Dap(Pyr)RTGRRFSYP-amide	64%
P6	Pyr-ßAla-GRTGRRFSYP-amide	49%
P7	Pyr-Abu-GRTGRRFSYP-amide	47%
P8	Pyr-Ava-GRTGRRFSYP-amide	31%
P9	Pyr-Ahx-GRTGRRFSYP-amide	48%
P10	Pyr-Aoc-GRTGRRFSYP-amide	39%
P11	Pyr-miniPEG™-GRTGRRFSYP-amide	38%

Beer's Law Analysis. The fluorescence intensities of different concentrations of phosphorylated **P5** peptide (ranging from 0 to 1 μM and incubated with 10 μM 14-3-3τ and 100 mM Tris HCl pH 7.5) were determined in the presence of 12.5 μM Rose Bengal. The intensities were plotted against the peptide concentration and the data fit to a straight line (pink). The fit of the data with background correction (gray) is shown as a dotted line. The background was acquired by using a sample that had all the assay components except the fluorophore-peptide by using the "Acquire Background" mode in FeliX software (Photon Technology version 1.42). This background intensity was automatically subtracted from subsequent measurements by the software.

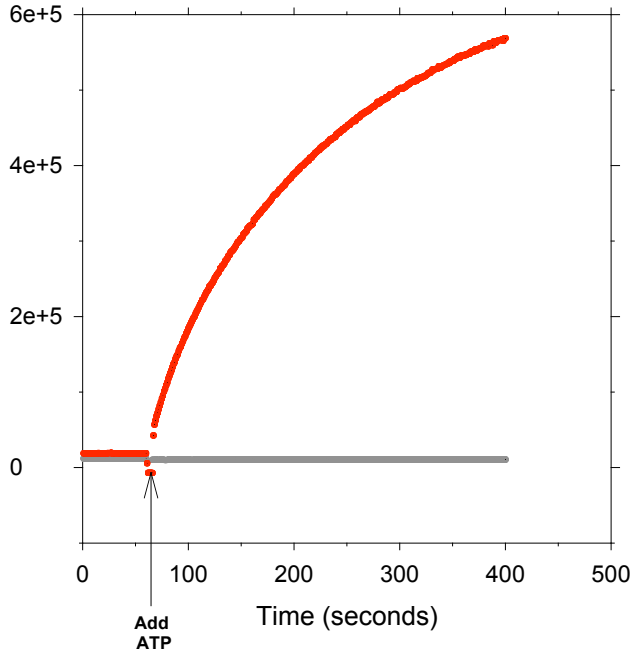
Figure S3. Fluorescence as a function of [peptide **P5**] before (magenta) and after (grey dotted) background correction.



Acquisition of K_m and V_{max} values: Phosphorylation dependent increase in pyrene fluorescence intensity of peptides **P2**, **P5** and **P9** were monitored on a Photon Technology QM-1 spectrofluorimeter at 30 ºC using 343 nm excitation wavelength, 380 nm emission wavelength, and an 8 nm slit-width. After equilibration of different concentrations of the pyrene-labeled peptide substrate with 50 mM Tris buffer pH 7.5, 30 μM 14-3-3τ, 1 mM ATP, 5 mM $MgCl_2$, 2 mM DTT, for 10 min, 10 nM enzyme was added and the reaction progress curves obtained. Reaction rates were determined from the slope under conditions where 5 - 8% substrate had been converted to product in duplicate. The resulting slopes (initial velocity, v_o) for each of the progress curves were plotted versus the concentration of substrate. A nonlinear regression analysis was used to fit the data to the rectangular hyperbola model using the Sigma Plot version 8.02 software.

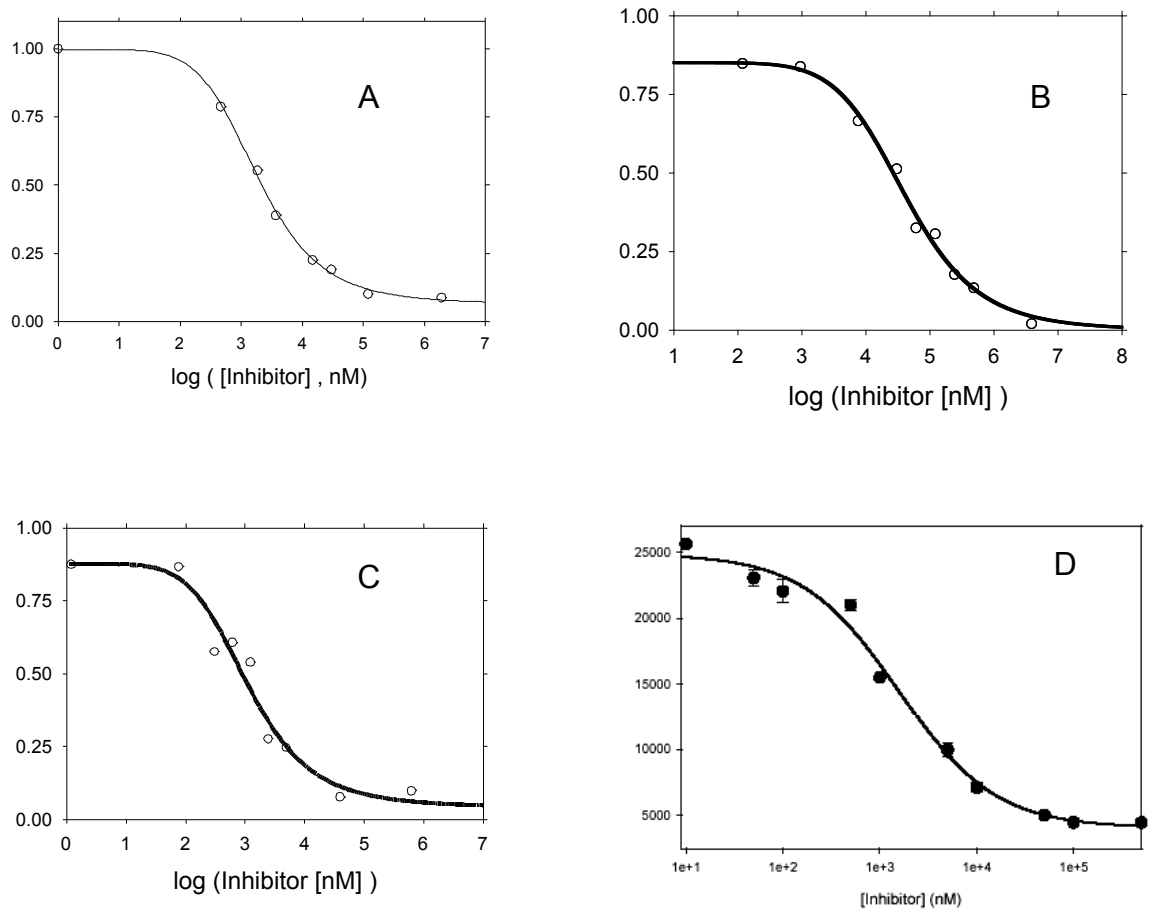
Assay Dependence on 14-3-3τ Phosphorylation-dependent increase in pyrene fluorescence intensity of peptide **P5**, in the presence and absence of 14-3-3τ, was monitored on a Photon Technology QM-1 spectrofluorimeter at 30 ºC using 343 nm excitation wavelength, 380 nm emission wavelength with an 8 nm slit-width. 5 μM pyrene-labeled peptide substrate **P5** was pre-incubated in 25 μM Rose Bengal, 5 mM $MgCl_2$, 2 mM DTT, 1.4 μM PKA, and 50 mM Tris buffer pH 7.5, in the presence (red), and absence (gray), of 30 μM 14-3-3τ, at 30 ^oC for 5 min. After 1 min, 1 mM ATP was added and the reaction progress followed. In the absence of 14-3-3τ (gray), no change in fluorescence intensity was observed.

Figure S4. PKA-induced fluorescence change of the Rose Bengal/peptide **P5** pair in the presence (red) and absence (grey) of 14-3-3τ.



Inhibitor IC_{50} values. 1 μM pyrene-labeled peptide substrate **P2** was incubated in 60 μM Ponceau S, 50 mM Tris buffer pH 7.5, 1 mM ATP, 30 μM 14-3-3τ, 5 mM $MgCl_2$, 2 mM DTT at 30 ^oC for 5 min. 10 nM PKA enzyme was added and the reaction progress followed for 1 min. This step was used to adjust for inter-assay variability and to verify that no significant drop in enzyme activity occurs over the course of the determinations. Subsequently, inhibitor was added at different concentrations. Reaction rates were measured under conditions where less than 10% substrate had been converted to product. Fractional velocities (v/v_0) were plotted against inhibitor concentration [I] and fit using the Sigma Plot version 8.02 software's four-parameter logistic nonlinear regression analysis. PKI (14-22) exhibits an IC_{50} value of 1.1 ± 0.1 μM. The IC_{50} value of PKI (14-22) using the standard radioactive ATP method is 1.6 ± 0.2 μM. H9•HCl exhibits an IC_{50} value of 42 ± 1 μM at 1 mM ATP and a value of 1.9 ± 0.2 μM at 10 μM ATP.

Figure S5. Fractional PKA activity versus log [inhibitor] for (A) H9•HCl at 10 μM ATP, (B) H9•HCl at 1 mM ATP, (C) PKI (14-22) using Deep Quench method, (D) PKI (14-22) using the standard radioactive ATP method.



Fluorescence change dependency on instrumentation/reading mode. We have found that the phosphorylation-induced fluorescence change is dependent upon instrumentation and reading mode. In brief, the least dramatic changes are observed in a plate reader (Molecular Devices Spectra Max Gemini EM) using the bottom read mode (i.e. from below through the bottom of a Hellma black quartz 96 well plate. Note: analogous results were obtained using a Costar 3631 flat bottom 96 multiwell plate). A more robust change is obtained via a top read mode with the Hellma black quartz 96 well plate (analogous results were obtained using a Wallac B & W isoplate 1450-582). The highest fluorescence fold change is provided using a dedicated spectrofluorimeter (Photon Technology QM-1) and a quartz cuvette as the sample holder. These results are summarized in Table S4 for the three lead peptide/quencher pairs. Initial screening of the library of peptides **P1** – **P11** with the ten lead quenchers (Chart S1) was performed using the bottom read mode. A summary of these results is furnished in Figure S6.

An example of assay conditions in plate reader mode is furnished for the top read with peptide **P5** and Rose Bengal: Phosphorylation-dependent increase in pyrene fluorescence intensity of peptide dye pair **P5**/Rose Bengal, was monitored on a Molecular Devices Spectra Max EM plate reader at 30 ºC using 343 nm excitation wavelength, 390 nm emission wavelength. Three wells containing 5 μM pyrene-labeled peptide substrate **P5** were pre-incubated in 5 mM $MgCl_2$, 2 mM DTT, 1 mM ATP, and 50 mM Tris buffer pH 7.5, 30 μM 14-3-3τ and 25 μM Rose Bengal, at 30 ^{o}C for 10 min. 0.7 μM PKA was added and the reaction progress followed. Three additional wells containing all the assay components except for **P5** peptide were used for blank readings. Note: for bottom read multiwell plate mode and single read cuvette mode a 343 nm excitation wavelength and 380 nm emission wavelength was employed.

Table S4. Phosphorylation-induced fluorescence fold-change of lead peptide/quencher pairs as a function of instrumentation, plate (P = plastic and Q = quartz), and read mode.

Peptide/Quencher (ratio)	Conditions	Fluorescence Change
P2/ Ponceau S (1:50)	Plate reader (P) – bottom read	7-fold
	Plate reader (Q) – bottom read	6-fold
	Plate reader (P) – top read	15-fold
	Plate reader (Q) – top read	7-fold
	spectrofluorimeter	21-fold
P5/Rose Bengal (1:5)	Plate reader (P) – bottom read	8-fold
	Plate reader (Q) – bottom read	24-fold
	Plate reader (P) – top read	30-fold
	Plate reader (Q) – top read	33-fold
	spectrofluorimeter	64-fold
P9/Aniline Blue WS (1:10)	Plate reader (P) – bottom read	9-fold
	Plate reader (Q) – bottom read	13-fold
	Plate reader (P) – top read	19-fold
	Plate reader (Q) – top read	28-fold
	spectrofluorimeter	55-fold

Figure S6. PKA-induced fluorescence change of peptide **P1** – **P11** in the presence of the ten lead quencher dyes. Peptide concentration was fixed at 5 μM and a 5-, 10-, 25-, and 50-fold excess of quencher was employed. Color scheme: light green (1.5 to <3-fold change), yellow (3< to <4.8-fold change), peach (4.8< to <6-fold change), orange (6< to <8-fold change), and red (8-fold change and above).

Peptide												
Dye	Fold excess dye	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
	50	1.15	0.94	0.99	0.91	0.78	0.70	0.81	0.88	0.83	0.73	0.74
D3	25	1.46	1.01	1.06	0.89	0.84	0.06	0.45	0.89	0.26	0.59	0.25
Evans Blue	10	1.43	1.37	1.06	0.91	0.92	0.74	0.31	0.36	0.24	0.64	0.12
	5	1.69	1.92	1.10	0.98	1.33	1.50	1.01	1.34	1.09	0.69	0.46
	50	3.85	6.30	1.68	4.27	5.79	4.70	5.20	5.01	4.03	3.48	2.46
D6	25	2.92	5.66	3.56	3.55	6.44	4.78	4.43	5.33	4.95	3.95	3.09
Reactive Blue	10	1.57	2.08	2.29	2.66	3.99	2.33	3.68	3.40	3.61	3.80	1.72
	5	1.00	1.99	1.48	2.16	2.28	1.63	2.64	2.57	3.39	5.12	1.57
	50	1.10	3.71	0.31	0.59	1.12	1.52	0.48	0.45	0.65	0.72	0.30
D18	25	0.41	1.13	0.58	0.59	0.84	0.87	1.58	0.62	1.46	0.79	0.56
Eriochrome Black T	10	1.13	1.42	1.02	1.12	1.73	1.37	1.64	1.47	1.52	2.23	0.71
	5	0.96	1.46	1.10	1.32	1.61	1.37	1.82	1.65	2.09	2.51	1.42
	50	1.13	1.30	1.05	1.07	1.50	1.27	1.34	1.64	2.03	2.44	1.14
D19	25	1.01	1.37	1.17	1.23	1.49	1.32	1.60	1.59	1.91	1.95	1.28
Alizarin Red	10	0.96	1.38	1.21	1.33	1.59	1.27	1.63	1.57	1.98	1.90	1.35
	5	0.98	1.59	1.28	1.36	1.70	1.35	1.71	1.68	2.01	1.97	1.31
	50	1.55	1.83	0.99	1.40	1.23	1.28	1.39	1.35	1.28	1.09	1.63
D27	25	4.13	2.52	3.79	5.58	6.04	5.60	4.76	1.81	3.59	2.39	2.21
Aniline Blue WS	10	0.47	2.48	2.17	4.82	5.36	3.48	6.63	6.22	8.94	6.77	1.78
	5	1.16	1.85	1.50	2.34	3.02	2.35	3.85	4.07	6.22	6.82	1.62
	50	0.10	0.35	0.25	0.87	0.25	0.38	0.95	0.97	0.85	0.89	0.19
D34	25	0.49	0.60	0.92	0.55	0.21	1.10	1.08	1.05	0.88	0.74	0.86
Chlorazol Black E	10	2.44	2.84	0.71	0.93	1.52	2.32	2.49	2.83	1.61	1.32	0.83
	5	1.42	2.26	1.79	4.01	5.62	2.27	4.81	6.65	3.63	4.80	1.62
	50	3.37	6.57	2.88	2.69	4.36	1.77	1.27	3.26	4.02	2.81	2.38
D42	25	1.84	4.61	2.75	2.88	3.23	3.13	2.19	2.01	4.64	5.66	2.12
Ponceau S	10	1.76	1.92	2.26	2.63	3.30	3.36	1.92	3.86	4.83	7.99	2.06
	5	1.35	1.93	1.81	2.26	2.85	2.77	2.00	3.14	4.32	7.56	2.10
	50	0.96	0.72	0.97	0.91	0.95	0.94	0.85	0.95	0.98	0.89	0.84
D43	25	1.03	1.58	1.13	1.10	1.02	1.20	0.85	0.81	1.11	1.23	0.32
Rose Bengal (Cert)	10	2.63	2.87	1.98	2.88	3.45	3.28	1.12	2.19	1.85	1.32	0.94
	5	1.20	2.58	2.70	6.52	8.36	2.71	7.44	5.43	3.51	3.26	3.54
	50	1.31	2.51	1.02	1.47	2.05	1.25	1.65	1.79	1.53	1.92	1.43
D47	25	1.16	1.82	1.47	1.46	1.79	1.42	1.77	1.58	1.82	1.76	1.28
Tartrazine	10	1.06	1.61	1.34	1.42	1.52	1.27	1.55	1.47	1.57	1.64	1.17
	5	0.99	1.41	1.19	1.28	1.43	1.23	1.46	1.39	1.55	1.47	1.15
D48	50	2.60	6.03	1.60	2.76	3.48	3.34	5.62	3.52	2.62	3.39	3.17
Trypan Blue	25	3.40	5.11	1.91	1.72	3.61	3.05	4.10	4.03	3.67	3.53	2.45
	10	3.04	2.81	2.72	2.18	2.72	2.17	4.76	4.87	3.96	3.45	3.40
	5	1.30	2.20	2.23	3.03	6.21	3.54	7.15	6.14	7.18	7.22	3.06

Figure S7. Assay of the cGMP-dependent protein kinase (PKG). **P2** peptide (10 μM) was pre-incubated with 50 mM Tris buffer pH 7.5, 30 μM 14-3-3τ, 1 mM ATP, 5 mM $MgCl_2$, 2 mM DTT, 60 μM Ponceau S dye with (red) and without (green) cGMP (1 mM) at 30ºC for 10 min. After 2 min, PKG (90 nM) was added and the reaction progress monitored. The initial rate v_o, (obtained from the 300 – 400 s interval of the progress-curve), is 8.2 fold faster in the presence than in the absence of cGMP.

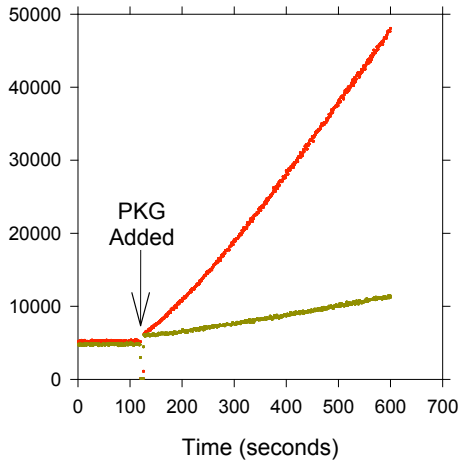


Figure S8. Effect of quencher on enzyme reaction rate. PKA concentration required to convert all substrate to product in 30 min is dependent upon the apparent K_d of quencher and substrate. The stronger the interaction between quencher and substrate the more enzyme required to achieve complete conversion of substrate to product in 30 min. This is consistent with the notion that the negatively charged quencher associates with the positively charged peptide substrate via electrostatic interactions. The positively charged residues on the peptide are required for recognition by PKA.