Electronic Supplementary Information (ESI)

Crosslinking of DNA-linked ligands to target proteins for enrichment from DNA-encoded libraries

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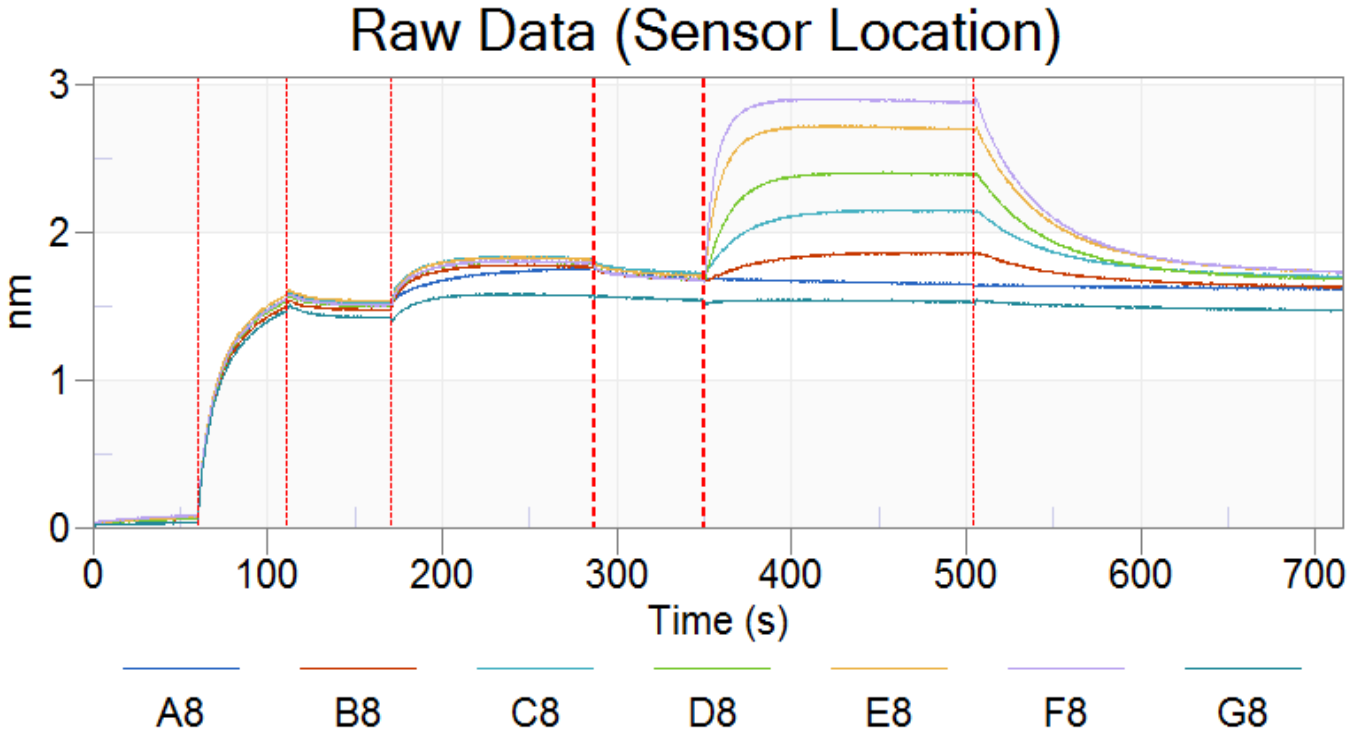
OctetRed384 data for DNA-ligand binding to CAII

General procedure for determining on-DNA K_d's:

SAX biosensors were pre-incubated in buffer (PBST (0.1 M sodium phosphate, pH 7.4, 0.15 M NaCl, 0.02% tween-20)) for 10 minutes. A reference well (no target protein) was used in all experiments and were performed at 30 °C

Baseline 1: (buffer) 60 s
Loading 1: (1 µM ssDNA'-5'-biotin in buffer) approx. 30 s or until 80 - 90 % signal saturation
Baseline 2: (buffer) 60 s
Loading 2: (0.5-5 µM 5'-ligand-ssDNA in buffer) approx. 120 s or until signal stabilizes
Baseline 3: (buffer) 60 s
Association: (varying CAII concentrations) 300 s or until signal stabilized
Dissociation: (buffer) 300 s or until signal returns or baseline or > 10 % decreased

Example raw data output:



Biosensor A8: no CAII
Biosensor B8, C8, D8, E8, F8: increasing concentrations of CAII
Biosensor G8: non-ligand DNA in 20 µM CAII

GL-CBS-ssDNA to CAII

Local fitting

Conc. (nM)	KD (M)	KD Error	kon(1/Ms)	kon Error	kdis(1/s)	kdis Error	Full X^2	Full R^2
62.5	4.91E-06	1.13E-05	1.05E+03	2.41E+03	5.14E-03	3.99E-05	0.2643	0.983
125	8.86E-08	7.42E-10	4.66E+04	3.80E+02	4.13E-03	7.97E-06	0.059	0.9986
250	1.23E-07	6.79E-10	3.70E+04	1.96E+02	4.54E-03	7.13E-06	0.0662	0.9992
500	9.51E-08	7.00E-10	4.03E+04	2.80E+02	3.83E-03	9.40E-06	0.3311	0.9977
1000	1.48E-07	7.69E-10	3.01E+04	1.49E+02	4.45E-03	7.23E-06	0.1667	0.9991

Global fitting

KD (M)	KD Error	kon(1/Ms)	kon Error	kdis(1/s)	kdis Error	Full X^2	Full R^2
1.21E-07	6.89E-10	3.53E+04	1.92E+02	4.26E-03	7.15E-06	2.4115	0.9965

Table 1 GL-CBS-ssDNA to CAII

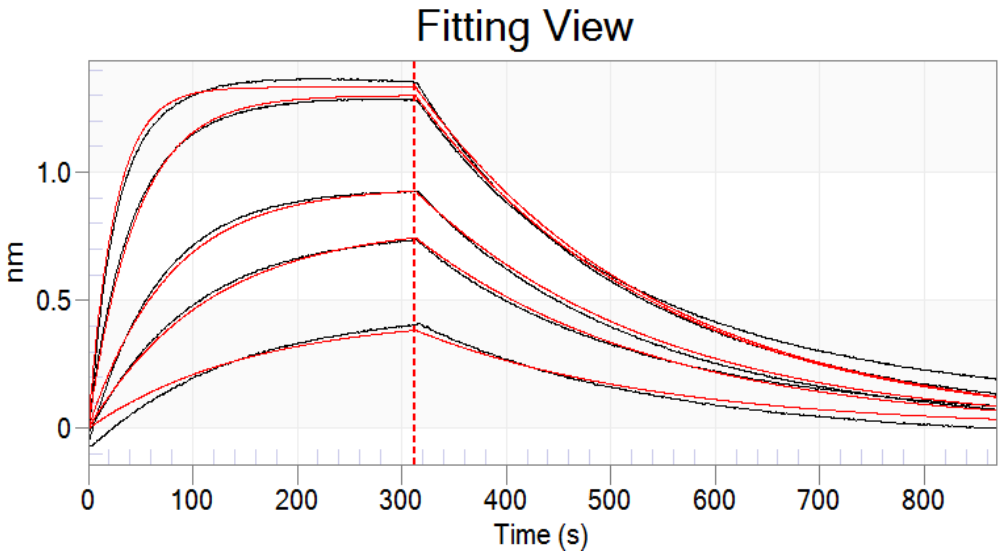


Figure S1 Binding curves with curve fits (red) of GL-CBS-ssDNA binding to CAII with reference well subtraction

CBS-ssDNA to CAII

Local fitting

Conc. (nM)	KD (M)	KD Error	kon(1/Ms)	kon Error	kdis(1/s)	kdis Error	Full X^2	Full R^2
259	2.10E-05	4.71E-05	8.10E+02	1.82E+03	1.75E-04	3.99E-05	0.0234	0.9911
778	9.72E-07	1.57E-08	1.49E+04	2.36E+02	4.62E-05	7.97E-06	0.0144	0.9989
2330	1.03E-06	1.43E-08	1.31E+04	1.73E+02	5.80E-05	7.13E-06	0.0746	0.998
7000	1.25E-06	2.21E-08	1.02E+04	1.71E+02	7.01E-05	9.40E-06	0.2717	0.9966
2.10E+04	2.45E-06	3.69E-08	5.89E+03	8.52E+01	6.11E-05	7.23E-06	0.1897	0.9983

Global fitting

KD (M)	KD Error	kon(1/Ms)	kon Error	kdis(1/s)	kdis Error	Full X^2	Full R^2
1.57E-06	1.81E-08	8.89E+03	9.88E+01	1.40E-02	4.36E-05	1.3159	0.9963

Table 2 CBS-ssDNA to CAII

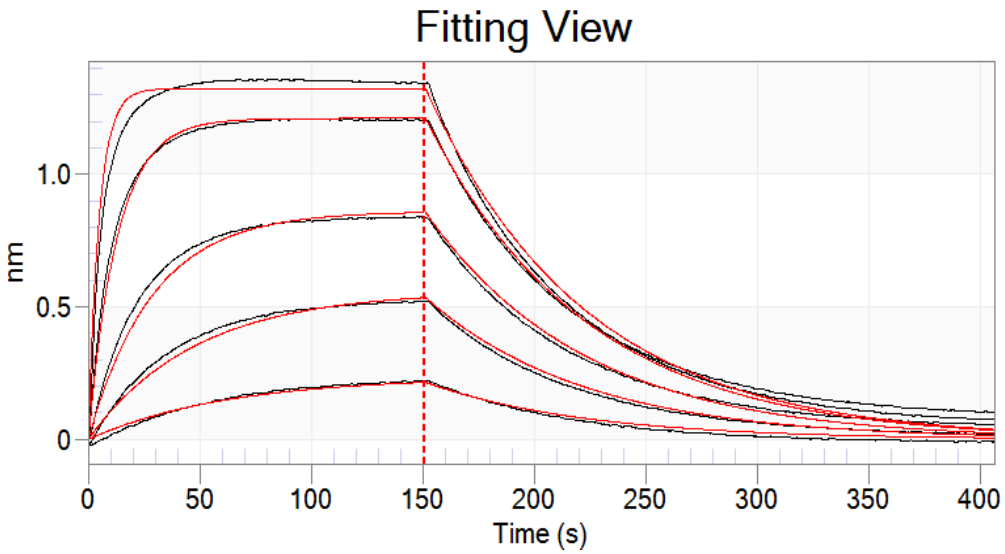


Figure S2 Binding curves with curve fits (red) of CBS-ssDNA binding to CAII with reference well subtraction

D-CBS-ssDNA to CAII

Local fitting

Conc. (nM)	KD (M)	KD Error	kon(1/Ms)	kon Error	kdis(1/s)	kdis Error	Full X^2	Full R^2
740	1.28E-06	4.36E-08	1.36E+04	4.56E+02	1.74E-02	1.03E-04	0.0072	0.9968
2220	8.08E-07	3.17E-08	1.63E+04	6.04E+02	1.31E-02	1.69E-04	0.2075	0.9798
6670	2.55E-06	6.98E-08	7.04E+03	1.82E+02	1.79E-02	1.60E-04	0.1975	0.9934
2.00E+04	3.87E-06	1.38E-07	4.49E+03	1.52E+02	1.74E-02	1.94E-04	0.6404	0.9893
6.00E+04	9.53E-06	3.51E-07	1.99E+03	7.01E+01	1.90E-02	2.12E-04	0.8141	0.9906

Global fitting

KD (M)	KD Error	kon(1/Ms)	kon Error	kdis(1/s)	kdis Error	Full X^2	Full R^2
4.67E-06	1.10E-07	3.90E+03	8.84E+01	1.82E-02	1.17E-04	3.3632	0.9876

Table 3 D-CBS-ssDNA to CAII

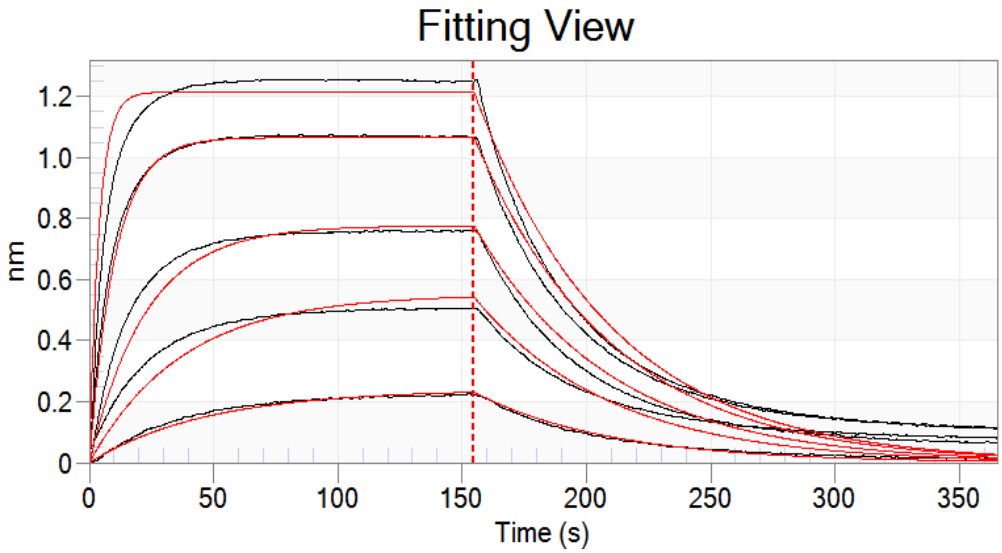
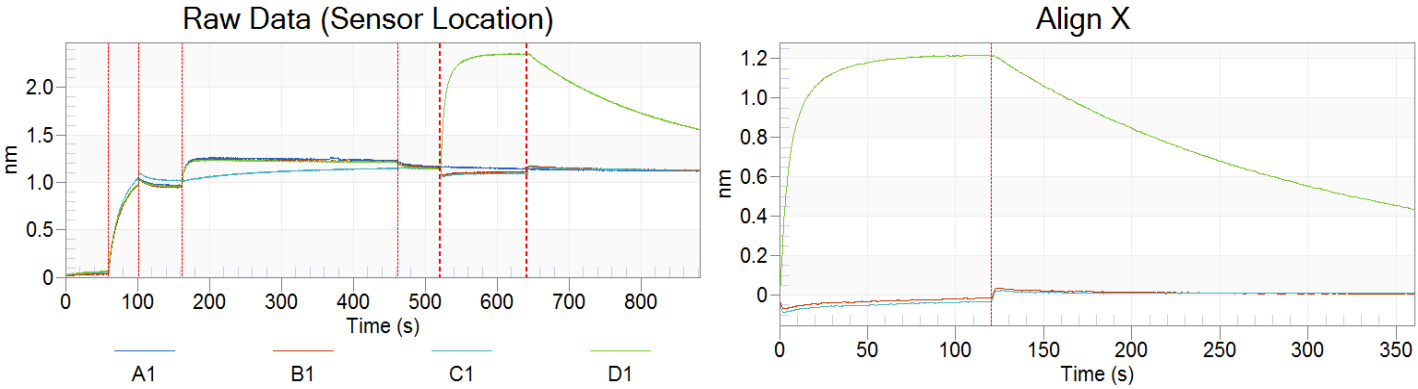


Figure S3 Binding curves with curve fits (red) of D-CBS-ssDNA binding to CAII with reference well subtraction

OctetRed384 data for DNA-GL-CBS binding to BSA



Biosensor A1: GL-CBS-ssDNA with no protein (reference well)

Biosensor B1: GL-CBS-ssDNA with 10 µM BSA

Biosensor C1: Bz-ssDNA with 10 µM BSA

Biosensor D1: GL-CBS-ssDNA with 10 µM CAII

Figure S4 Binding curves of D-CBS-ssDNA binding to BSA or CAII with reference well subtraction

Crosslinking of CBX8

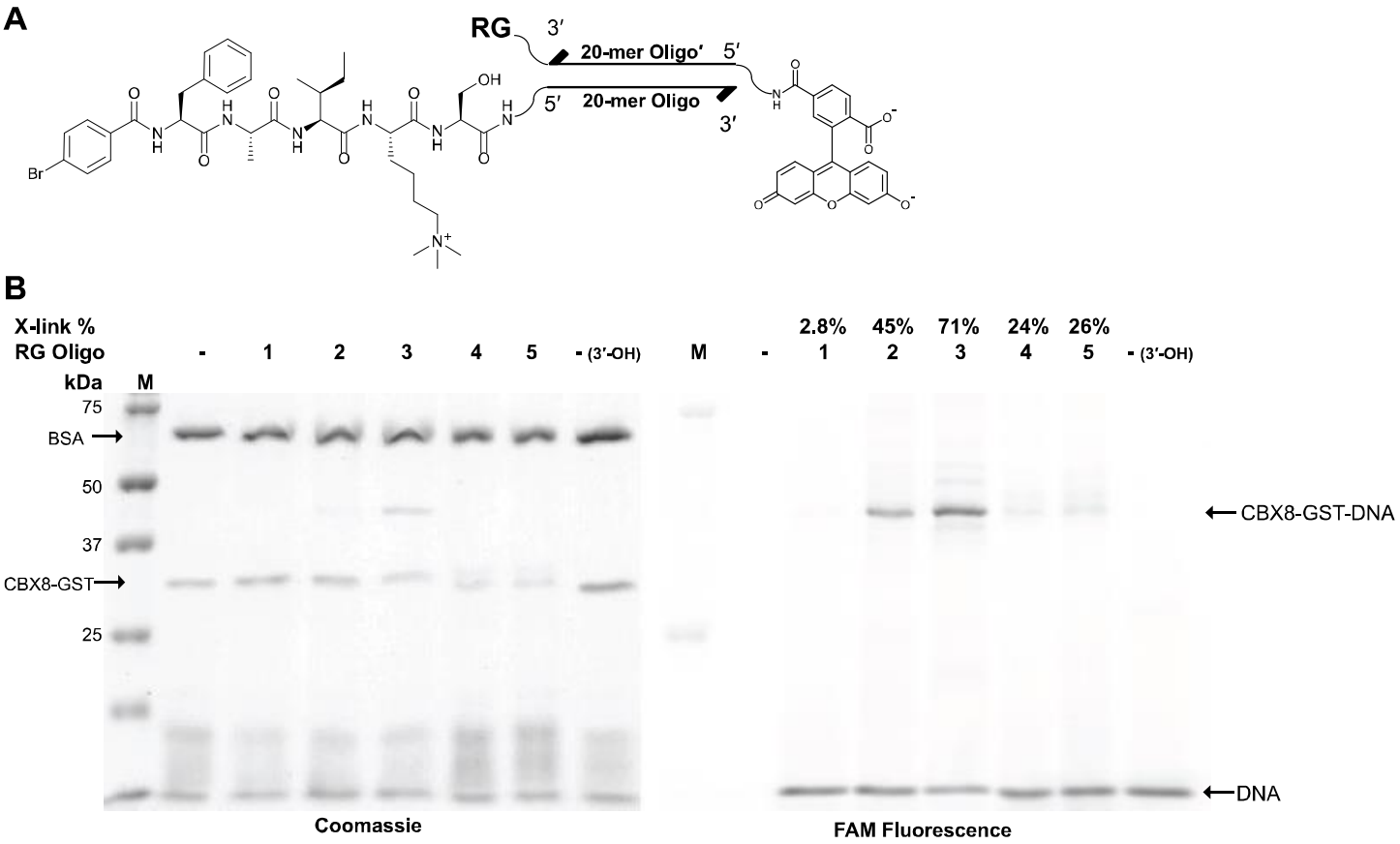


Figure S5 Ligand-directed crosslinking of DNA to CBX8-GST with various reactive groups. (A) Structure of CBX peptide ligand-ssDNA. (B) Reactive group labels as given in main text Fig. 1 (tosyl **1**, NHS ester **2**, sulfonyl fluoride **3**, phenyl azide **4**, diazirine **5**). A non-reactive group-containing oligo (ssDNA'-5'-FAM) (5'-OH ssDNA') was used as a non-reactive control in addition to the non-ligand (5'-OH ssDNA) control (**-**). Buffer: 20 mM HEPES, pH 7.4, 0.25 M NaCl, 0.02% (v/v) tween-20, 1.0 mg/mL tRNAs with 1.0 µM CBX8-GST, 1.0 M BSA, 1.0 µM ligand-ssDNA, 0.75 µM RG-ssDNA'.

Crosslinking of PKA

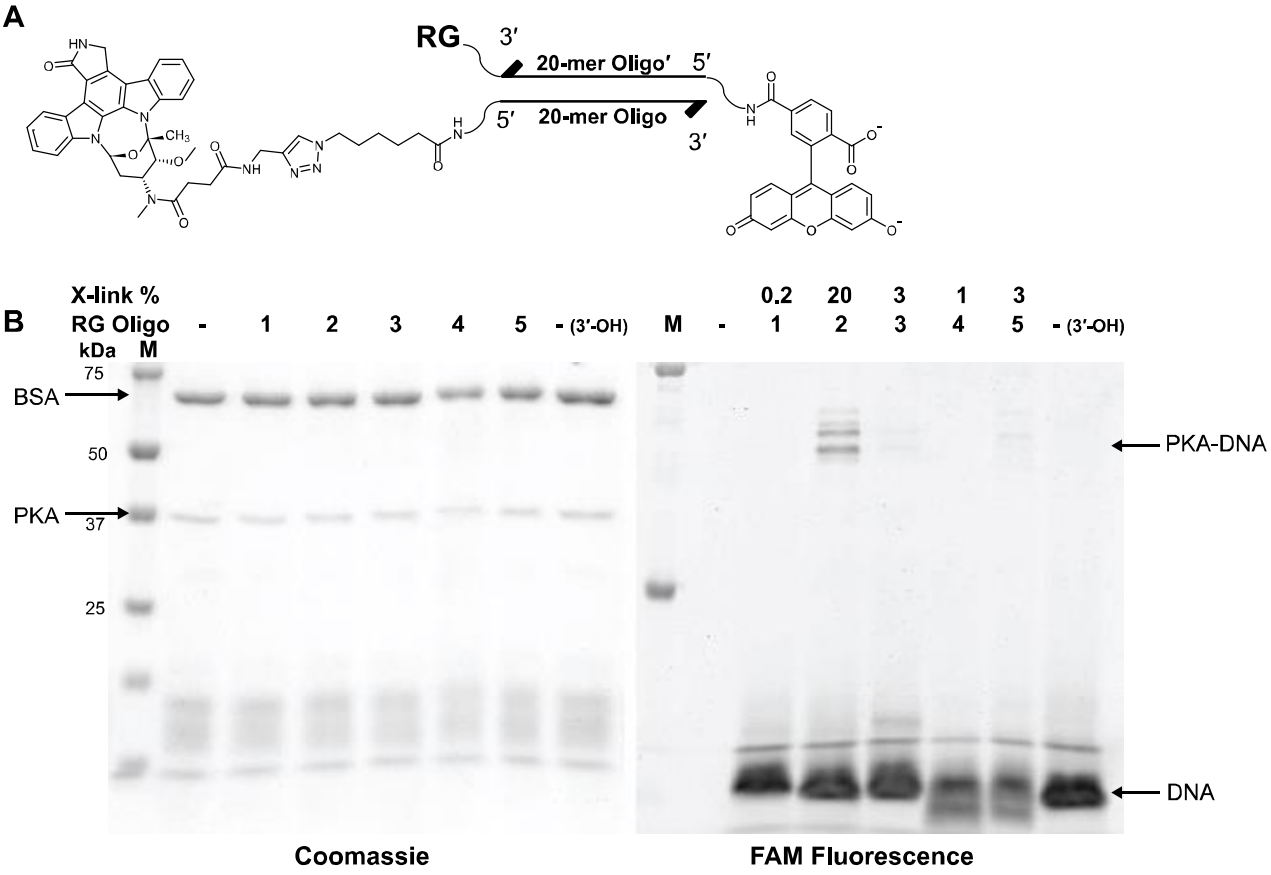


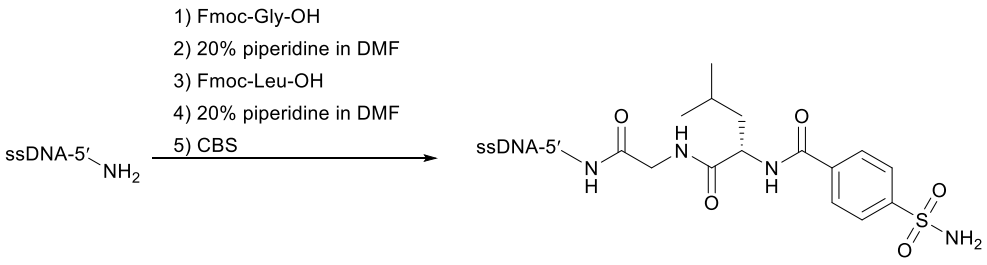
Figure S6 Ligand-directed crosslinking of DNA to PKA (catalytic subunit) with various reactive groups. (A) Structure of STS-ssDNA. (B) Reactive group labels as given in main text Fig. 1 (tosyl **1**, NHS ester **2**, sulfonyl fluoride **3**, phenyl azide **4**, diazirine **5**). A non-reactive group-containing oligo (ssDNA'-5'-FAM) (5'-OH ssDNA') was used as a non-reactive control in addition to the non-ligand (5'-OH ssDNA) control (**-**). Buffer: 20 mM HEPES, pH 7.4, 0.25 M NaCl, 10 mM $MgCl_2$, 0.02% (v/v) tween-20, 1.0 mg/mL tRNAs with 1.0 µM CBX8-GST, 1.0 µM BSA, 1.0 µM ligand-ssDNA, 0.75 µM RG-ssDNA'.

Oligonucleotide sequences and modifications

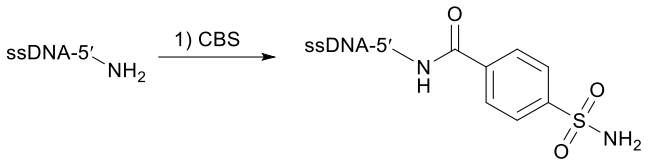
ssDNA-5′-C12-NH_2	/5AmMC12/ATGGTATCAAGCTTGCCACA
ssDNA-5′-PEG-NH_2	/5AmMC6/5Sp18/ATGGTATCAAGCTTGCCACA
ssDNA′-3′-NH_2	TGTGGCAAGCTTGATACCAT/3AmMO/
ssDNA′-3′-NH_2-5′-FAM	/56-FAM/TGTGGCAAGCTTGATACCAT/3AmMO/
ssDNA-linker-ssDNA-5′-NH_2	/5AmMC12/ATGGTATCAAGCTTGCCACA/iSp9/GTCGAGCTCTCTACTGCATA
ssDNA′-5′-FAM	/56-FAM/TGTGGCAAGCTTGATACCAT
ssDNA′-5′-Biotin	/5Biosg/TGTGGCAAGCTTGATACCAT

Abbreviations for modifications within sequences are given as used by Integrated DNA Technologies (IDT).

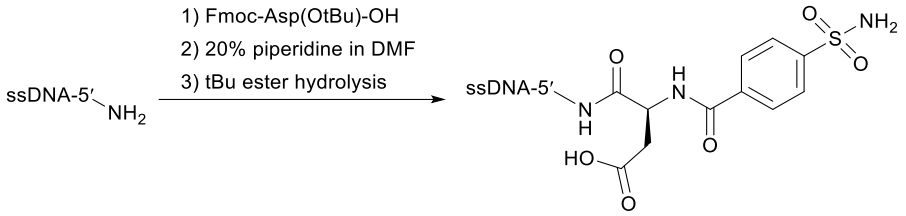
Synthesis of ligands on DNA



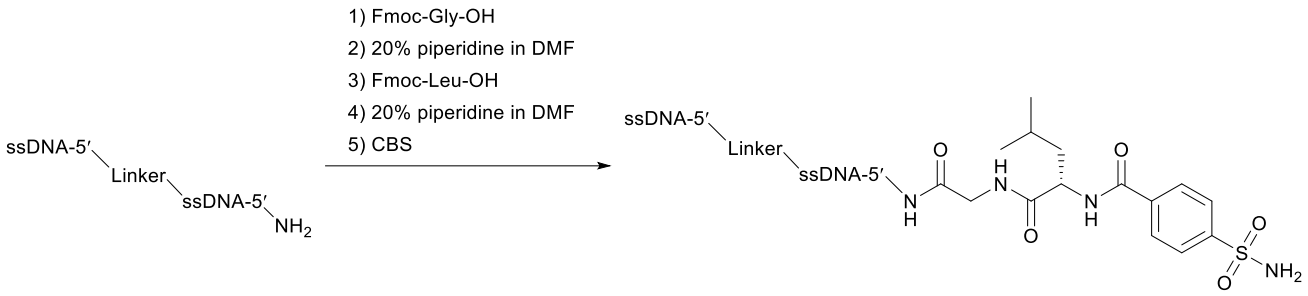
GLCBS-ssDNA was prepared by a modified acylation conditions described in the main text[1]. Briefly, the 5´-amine modified ssDNA was immobilized on DEAE sepharose in a solid phase synthesis cartridge with DEAE bind buffer and then equilibrated in MeOH. The acylation reaction mixture (50 mM Fmoc-Gly-OH, 50 mM EDC-HCl, 5 mM HOAt in 40:60 DMF:MeOH) was incubated for 30 minutes. The reaction mixture was then eluted and a fresh acylation reaction mixture was prepared and incubated again for 30 minutes at RT. The reaction mixture was eluted and the resin was washed with MeOH and then DMF. Deprotection of the Fmoc protecting group was completed by incubating the cartridge in 20 % piperidine in DMF for 30 minutes at RT. The deprotection mixture was eluted and the resin washed with DMF and then MeOH. Fmoc-L-Leu-OH was then coupled and deprotected using the sample procedure. The resulting dipeptide-ssDNA conjugate was capped with 4-carboxybenzenesulfonamide (CBS) by preparing a mixture of 50 mM CBS, 50 mM EDC-HCl, 5 mM HOAt in MeOH and incubating the cartridge for 30 minutes at RT. The acylation was repeated, and then the resin was washed with MeOH and DEAE bind buffer. The modified oligo was eluted by passing 1 mL of DEAE elution buffer through the cartridge. The resulting mixture was directly purified by HPLC. ESI: $(M-11H)^{11-}$ 609.7 (calcd. 609.7), $(M-10H)^{10-}$ 607.7 (calcd. 670.8), $(M-9H)^{9-}$ 745.4 (calcd. 745.4), $(M-8H)^{8-}$ 838.8 (calcd. 838.7), $(M-7H)^{7-}$ 958.7 (calcd. 958.7), $(M-6H)^{6-}$ 1118.8 (calcd. 1118.6).



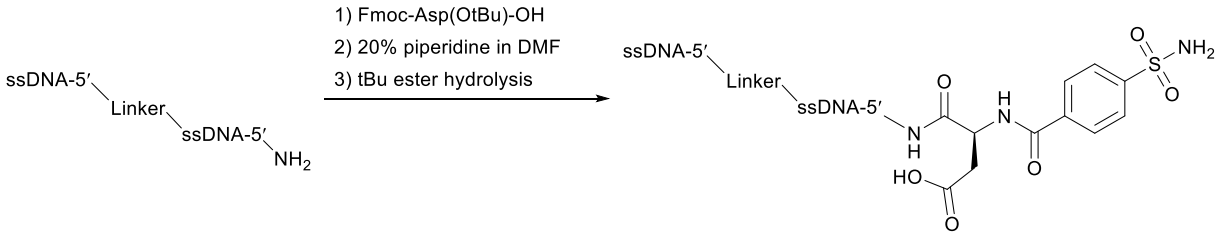
CBS-ssDNA was prepared using a similar procedure as GL-CBS-ssDNA. A double coupling using CBS was completed on ssDNA-5´-PEG-NH_2 and the resulting conjugate was eluted and directly purified by HPLC. MALDI: $(M-H)^-$ 6807.7 (calcd. 6807.0).



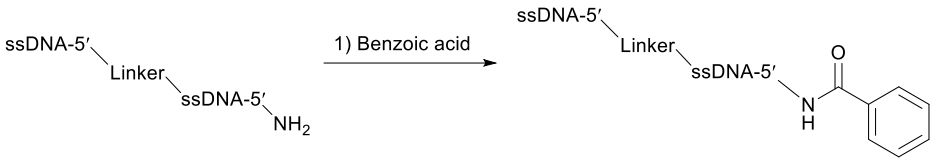
D-CBS-ssDNA was prepared using a similar procedure as CBS-ssDNA. To ssDNA-5´-PEG-NH_2, a double coupling was completed using Fmoc-Asp(OtBu)-OH, followed by Fmoc deprotection and capping with CBS. The resulting conjugate was eluted in tBu ester DEAE elution buffer (1.5 M NaCl, 0.2 M $MgCl_2$, 100 mM TEAA, pH 5.5, 0.005 % Triton X-100). The elution mixture was heated at 65 °C for 24 h and then directly HPLC purified. MALDI: $(M-H)^-$ 6923.7 (calcd. 6921.8).



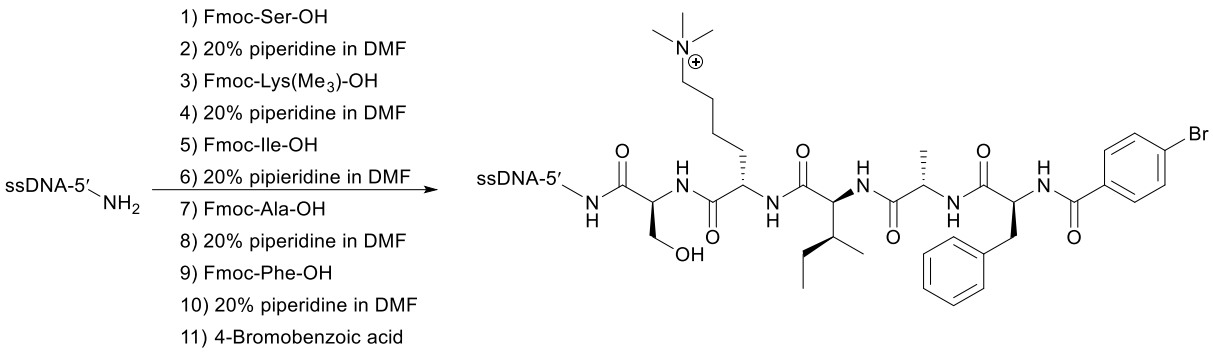
GL-CBS-ssDNA-linker-ssDNA was prepared using a similar procedure as GLCBS-ssDNA, except using ssDNA-linker-ssDNA-5´-NH_2 as the amine-modified oligo. The eluted oligo was directly HPLC purified. MALDI: $(M-H)^-$ 13,188.63 (calcd. 13,191.1), $(M-2H)^{2-}$ 6596.76 (calcd. 6595.1).



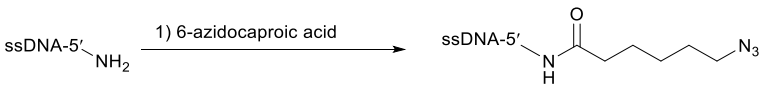
D-CBS-ssDNA-linker-ssDNA was prepared using a similar procedure as D-CBS-ssDNA, except using ssDNA-linker-ssDNA-5´-NH_2 as the starting oligo. The eluted oligo was directly HPLC purified. MALDI: $(M+Na-2H)^-$ 13,158.3 (calcd. 13,157.9).



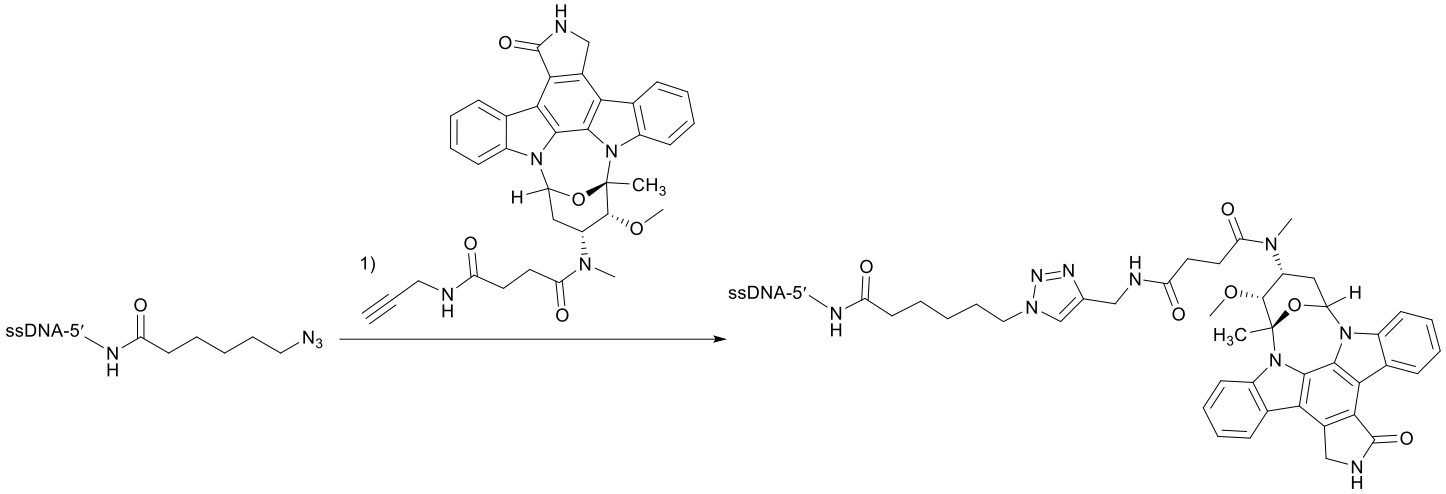
Bz-ssDNA-linker-ssDNA was prepared using a similar procedure as D-CBS-ssDNA-linker-ssDNA. To ssDNA-linker-ssDNA-5´-NH_2, a double coupling with benzoic acid was completed and the resulting conjugate was eluted and directly purified by HPLC. MALDI: $(M+H)^+$ 12,948.6 (calcd. 12,941.7).



CBX peptide ligand-ssDNA (ssDNA-5´-PEG-SK(me_3)IAF-4BrBa) was prepared using a modified procedure as ssDNA-5´-GL-CBS. All amino acids were double coupled, for 30 minutes at RT each coupling, using 50 mM Fmoc-AA (Fmoc-L-Ser(OH)-OH, Fmoc-L-Lys(Me_3)-OH, Fmoc-L-Ile-OH, Fmoc-L-Ala-OH, Fmoc-L-Phe-OH), 50 mM EDC-HCl, and 5 mM HOAt in 40:60 DMF:MeOH, with the exception of Fmoc-L-Ile-OH which was completed using 50 mM EDC-HCl, and 50 mM HOAt in 40:60 DMF:MeOH and was triple coupled. Fmoc deprotections were completed in 40% piperidine in DMF, 30 minutes at RT. The peptide was capped with 4-bromobenzoic acid (4BrBA) using 50 mM 4BrBa, 50 mM EDC-HCl, 5 mM HOAt in MeOH, for 30 minutes at RT and repeated. The resulting DNA-peptide conjugate was eluted and directly HPLC purified. ESI: $(M-12H)^{11-}$ 671.5 (calcd. 671.4), $(M-11H)^{10-}$ 738.7 (calcd. 738.6), $(M-10H)^{9-}$ 820.9 (calcd. 820.8), $(M-9H)^{8-}$ 923.6 (calcd. 923.5), $(M-8H)^{7-}$ 1055.6 (calcd. 1055.6), $(M-5H)^{6-}$ 1231.6 (calcd. 1231.7).

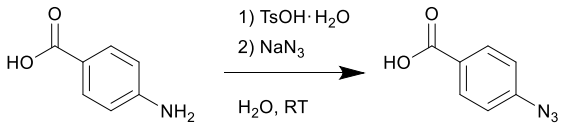


ssDNA-5′-CapN3 was prepared using the same procedure as ssDNA-5´-D-CBS. Briefly, a double coupling using 6-azidohexanoic acid was completed and the resulting conjugate was eluted and directly HPLC purified. MALDI: $(M-H)^-$ 6764.3 (calcd. 6763.7).

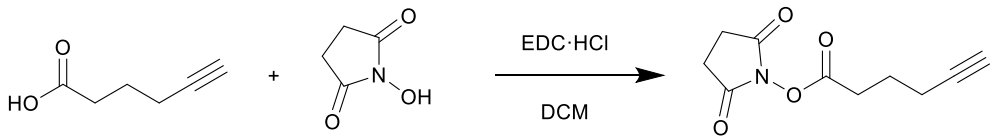


STS-ssDNA (ssDNA-5´-PEG-Cap-STS) was prepared as follows: the alkynyl staurosporine derivative was prepared as previously reported2. The crude product was used directly for conjugation to ssDNA-5'-CapN3 via CuAAC3. Briefly, 1 nmol of ssDNA-5'-CapN3 was suspended in 1.0 µL 2M TEAA, pH 6.5, 10.0 µL of crude alkynyl staurosporine in DMSO (approx. 100 eq.), 4.0 µL 50 mM THPTA. To this, 2.0 µL of a fresh solution of sat. CuBr in DMSO (approx 1 mg/1 mL) was added, mixed thoroughly, and incubated at RT for 3 h. 10 L of 0.5 M EDTA, pH 8.0 was added and directly HPLC purified. MALDI: (M+Na-2H)- 7353.4 (calcd. 7353.3).

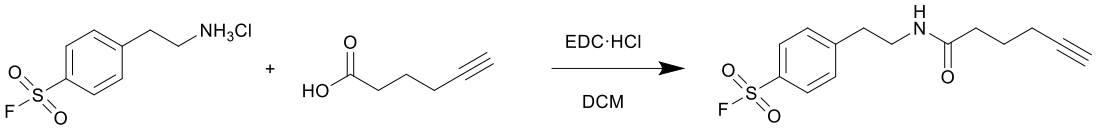
Synthesis of reactive groups



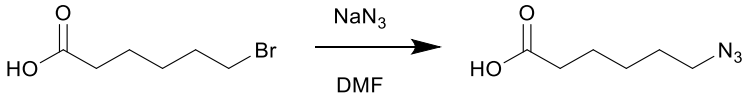
4-Azidobenzoic acid was prepared as previously described[4]. To 9 mL H_2O, 1.62 g (8.52 mmol, 8.52 eq.) *p*-toluenesulfonic acid monohydrate and 0.137 g (1.00 mmol, 1.00 eq.) 4-aminobenzoic acid and was added and stirred until dissolved. Then, 0.621 g (9.00 mmol, 9.00 eq.) sodium nitrite was added slowly over 5 minutes. The mixture was stirred at RT for 30 minutes, followed by the slow addition of 0.104 g (1.60 mmol, 1.60 eq.) resulting in an immediate release of N_2 and formation of a white ppt. The mixture was stirred for an additional 30 minutes then filtered, washed with water and dried, yielding 0.088 g (54 %) of white solid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.96 (d, *J* = 8.7 Hz, 2 H), 7.22 (d, *J* = 8.4 Hz, 2 H); CI-MS-CPI: (M+H) 164.20 (calcd. 164.05), (M-N_2+H) 136.20 (calcd. 136.04).



NHS 5-hexynoate was prepared as previously described[5]. To 200 mL DCM, 0.500 g (4.46 mmol, 1.00 eq.) 5-hexynoic acid was stirred with 0.538 g (4.68 mmol, 1.05 eq.) *N*-hydroxysuccinimide with 0.897 g (4.68 mmol, 1.05 eq.) EDC-HCl at RT for 7 hours. The reaction mixture was diluted and washed with H_2O, $NaHCO_3$ (aq), and sat. NaCl, then dried over $MgSO_4$ and concentrated to give 0.74 g (80 %) pale yellow oil. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 2.83 (s, 4H), 2.77 (t, *J* = 7.5 Hz, 2H), 2.34 (dt, *J* = 2.7, 6.9 Hz, 2H), 2.012 (t, *J* = 2.7 Hz, 1H), 1.96 (quint, *J* = 7.2 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 17.5, 23.3, 25.5, 29.6, 69.8, 82.0, 168.1, 169.0.



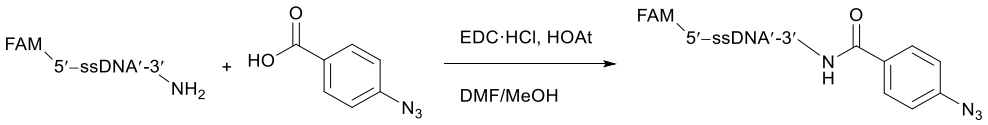
4-[2-(hex-5-ynoylamino)ethyl]benzenesulfonyl fluoride was prepared as follows: in 10 mL DCM, 0.460 g (2.40 mmol, 1.2 eq.) EDC-HCl and 700 µL (5.0 mmol, 2.5 eq.) DIEA was added with 110 µL (2.00 mmol, 1.0 eq.) 5-hexynoic acid and stirred for 5 minutes at 0 °C under Ar. To this, 0.575 g (2.4 mmol, 1.2 eq.) 4-aminomethylbenzenesulfonyl fluoride-HCl was added portionwise while stirring at 0 °C. The mixture was allowed to slowly warm to RT over 6 h. The reaction was then diluted with 100 mL DCM and washed 2x 100 mL half-saturated $NaHCO_3$ (aq), 3x 100 mL 0.1M HCl (aq), sat. NaCl (aq), and then dried over MgSO4 and concentrated *in vacuo* to give 0.384 g of white solid (69 %). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.95 (d, *J* = 8.4 Hz, 2H), 7.46 (d, *J* = 8.1 Hz, 2H), 5.58 (br s, 1H), 3.56 (q, *J* = 6.3 Hz, 2 H), 2.97 (t, *J* = 6.9 Hz, 2 H), 2.23 (t, *J* = 7.2 Hz, 2H), 2.22 (dt, *J* = 2.7, 6.9 Hz, 2 H), 1.96 (t, *J* = 2.7 Hz, 1 H), 1.83 (quint, *J* = 6.9 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.5, 148.0, 131.2, 130.2, 128.9, 83.4, 69.4, 40.2, 36.0, 34.6, 24.1, 17.8; CI-MS: $(M+H)^+$ 298.35 (calcd. 298.35), $(M-HF)^+$ 278.35 (calcd. 278.35).



6-Azidocaproic acid was prepared as previously described[6]. To 5 mL of DMF, 0.782 g (4.00 mmol, 1.0 eq.) 6-bromohexanoic acid was stirred with 0.520 g (8.00 mmol, 2.0 eq.) NaN_3 for 12 h at 85 °C. The reaction was cooled to RT then diluted with water and extracted with DCM. The combined organic phases were washed with 0.1 M HCl (aq), brine, and then dried over $MgSO_4$, and concentrated *in vacuo* to give 0.525 g (83 %) of yellow oil. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.28 (t, *J* = 6.6 Hz, 2H), 2.32 (t, *J* = 7.2 Hz, 2H), 1.62 (m, 4H), 1.42 (m, 2H).

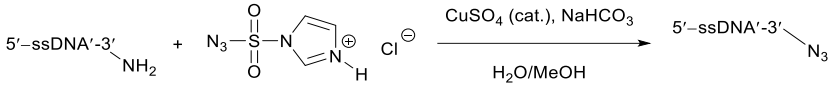
Synthesis of reactive groups on DNA

3′-Diazirine-5′-FAM-ssDNA′ (**5**) was prepared from ssDNA′-3′-NH_2-5′-FAM-ssDNA using 50 mM 3-Methyl-diazirine-3-propanoic acid, 50 mM EDC-HCl, 5 mM HOAt in 40:60 DMF:MeOH, 30 minutes at RT with double coupling. The conjugate was eluted in Alkali DEAE Elution Buffer (0.1 M Tris, pH 8.0, 1.5 M NaCl, 0.005 % Triton X-100) and directly purified by HPLC. MALDI: $(M-H)^-$ 7014.1 (calcd. 6,9893.7), $(M-2H)^{2-}$ 3498.9 (calcd. 3494.4). *Alkali conditions were used to reverse acylation of FAM prior to HPLC purification



3′-Phenyl azide-5′-FAM- ssDNA′ (**4**) was prepared from ssDNA′-3′-NH_2-5′-FAM-ssDNA using 4-Azidobenzoic acid was coupled onto ssDNA′-3′-NH_2-5′-FAM using the same procedure as for **5**. MALDI: $(M-H)^-$ 6965.7 (calcd. 7025.7). *MALDI laser (337 nm) can decompose PhN_3

ssDNA′-3′-CapN$_3$-5′-FAM was prepared by coupling 6-azidohexanoic acid to ssDNA′-3′-NH$_2$-5′-FAM using the same procedure as for **5**. MALDI: (M-H)$^-$ 7020.83 (calcd. 7018.8). *Diazotransfer conditions were attempted but were incompatible with the FAM moiety.



ssDNA′-3′-N$_3$ was prepared via diazotransfer of ssDNA′-3′-NH$_2$ was completed using the procedure described by Lartia et al.[7] Briefly, 20 nmol of ssDNA′-3′-N$_3$ was suspended in 65 µL 50 mM NaHCO3 in 3:1 H$_2$O:MeOH, with 1.2 µL 50 mM CuSO4 and 3.6 µL fresh imidazole-1-sulfonyl azide-HCl[8] in 50% MeOH (aq) and heated at 60 C for 2 h. The reaction was quenched by adding 1.2 L 0.5 M EDTA, pH 8.0 and directly HPLC purified. ESI: (M-11H)$^{11-}$ 578.0 (calcd. 578.0), (M-10H)$^{10-}$ 635.9 (calcd. 635.9), (M-9H)$^{9-}$ 706.7 (calcd. 706.7), (M-8H)$^{8-}$ 795.1 (calcd. 795.2), (M-7H)$^{7-}$ 908.8 (calcd. 908.9), (M-6H)$^{6-}$ 1060.2 (calcd. 1060.5).

References

1 D. R. Halpin, J. A. Lee, S. J. Wrenn and P. B. Harbury, *PLoS Biol.*, 2004, **2**, 1031-1038.

2 H. Shi, X. Cheng, S. K. Sze and S. Q. Yao, *Chem. Commun.*, 2011, **47**, 11306.

3 Jena Bioscience, *Copper(I) Catalyzed Click Reactions*, 2011, http://www.jenabioscience.com/images/741d0cd7d0/Copper_Catalyzed_Click_Reactions.pdf

4 K. V. Kutonova, M. E. Trusova, P. Postnikov, V. D. Filimonov and J. Parello, *Synth.*, 2013, **45**, 2706–2710.

5 B. Jagadish, R. Sankaranarayanan, L. Xu, R. Richards, J. Vagner, V. J. Hruby, R. J. Gillies and E. A. Mash, *Bioorganic Med. Chem. Lett.*, 2007, **17**, 3310–3313.

6 C. Bin Yim, O. C. Boerman, M. De Visser, M. De Jong, A. C. Dechesne, D. T. S. Rijkers and R. M. J. Liskamp, *Bioconjug. Chem.*, 2009, **20**, 1323–1331.

7 R. Lartia, P. Murat, P. Dumy and E. Defrancq, *Org. Lett.*, 2011, **13**, 5672–5675.

8 E. D. Goddard-Borger and R. V. Stick, *Org. Lett.*, 2007, **9**, 3797–3800.