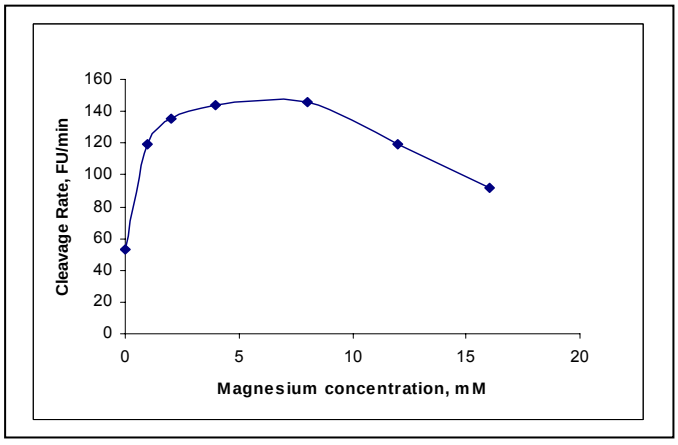
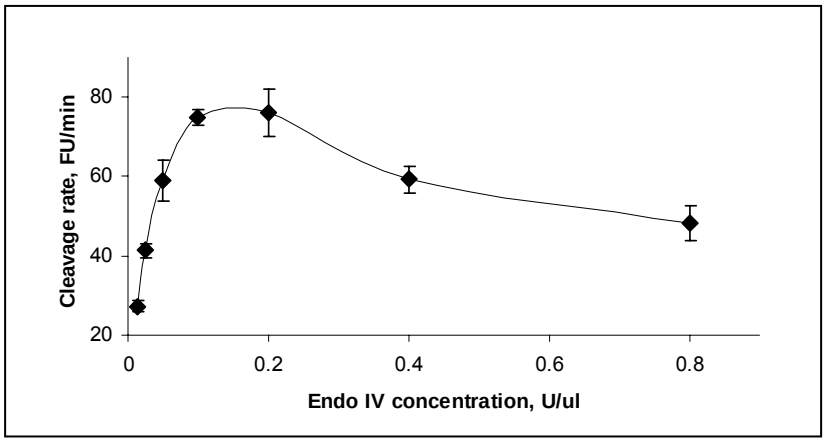
Nanogen, Inc.

SUPPLEMENTARY MATERIAL

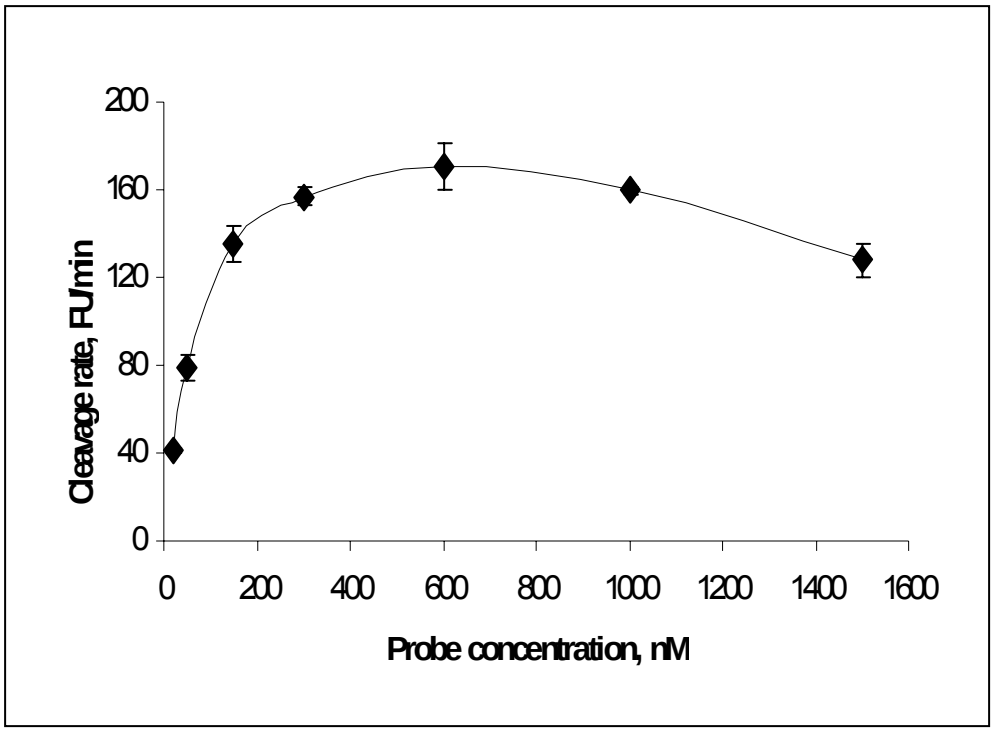
Supplementary Figure 1. Cleavage reaction was performed with the probe (150 nM) and enhancer (150 nM) and Endo IV (0.2 U/μl) in 20 mM Tris-HCl buffer (pH8.6) in varying $MgCl_2$ concentrations**.**



Supplementary Figure 2. Cleavage reaction was performed with the probe (150 nM), enhancer (150 nM), 5 mM $MgCl_2$) and varying concentrations of Endo IV in 20 mM Tris-HCl buffer (pH 8.6).



Supplementary Figure 3. Cleavage reaction was performed with the enhancer (1500 nM), 5 mM $MgCl_2$, 0.2 U/μl Endo IV and varying probe concentrations in 20 mM Tris-HCl buffer (pH8.6).



Supplementary Table 1. The probe and complementary target sequences indicating the mismatches, shown in bold.

	3'-5' Sequence
Probe	FL-CCGTTCCTGGCTCA-Q
Position / Mismatch	**5'-3' Sequence**
Matched	AGTCACAGTCGGTGCCAATGTGGCGGGCAAGGACCGAGTCG
1-C/A	AGTCACAGTCGGTGCCAATGTGGCG**A**GCAAGGACCGAGTCG
1-C/T	AGTCACAGTCGGTGCCAATGTGGCG**T**GCAAGGACCGAGTCG
1-C/C	AGTCACAGTCGGTGCCAATGTGGCG**C**GCAAGGACCGAGTCG
2-C/A	AGTCACAGTCGGTGCCAATGTGGCGG**A**CAAGGACCGAGTCG
2-C/T	AGTCACAGTCGGTGCCAATGTGGCGG**T**CAAGGACCGAGTCG
2-C/C	AGTCACAGTCGGTGCCAATGTGGCGG**C**CAAGGACCGAGTCG
3-G/G	AGTCACAGTCGGTGCCAATGTGGCGGG**G**AAGGACCGAGTCG
3-G/A	AGTCACAGTCGGTGCCAATGTGGCGGG**A**AAGGACCGAGTCG
3-G/T	AGTCACAGTCGGTGCCAATGTGGCGGG**T**AAGGACCGAGTCG

4-T/G	AGTCACAGTCGGTGCCAATGTGGCGGGC**G**AGGACCGAGTCG
4-T/C	AGTCACAGTCGGTGCCAATGTGGCGGGC**C**AGGACCGAGTCG
4-T/T	AGTCACAGTCGGTGCCAATGTGGCGGGC**T**AGGACCGAGTCG
5-T/G	AGTCACAGTCGGTGCCAATGTGGCGGGCA**G**GGACCGAGTCG
5-T/C	AGTCACAGTCGGTGCCAATGTGGCGGGCA**C**GGACCGAGTCG
5-T/T	AGTCACAGTCGGTGCCAATGTGGCGGGCA**T**GGACCGAGTCG
6-C/A	AGTCACAGTCGGTGCCAATGTGGCGGGCAA**A**GACCGAGTCG
6-C/C	AGTCACAGTCGGTGCCAATGTGGCGGGCAA**C**GACCGAGTCG
6-C/T	AGTCACAGTCGGTGCCAATGTGGCGGGCAA**T**GACCGAGTCG
8-T/G	AGTCACAGTCGGTGCCAATGTGGCGGGCAAGG**G**CCGAGTCG

3-(3-Chloro-2,4-dimethoxyphenyl)acrylic acid (2). *A* solution of 3-chloro-2,4-dimethoxybenzaldehyde (**1**) (Plattner, J. J. et al. J.Med.Chem. l984, 27(8), 1016-1026) (11.07 g, 55.2 mmol), malonic acid (8.639 g, 83 mmol) and piperidine (2 ml, 1.722 g, 20.2 mmol) in 80 ml of anhydrous pyridine was refluxed (bath temperature 110°C) for 2 h. Reaction mixture was cooled, concentrated under vacuum and acidified 250 ml of 5% aqueous citric acid to a pH of 3. Resulted heterogeneous mixture was sonicated for a few minutes. The precipitated material was filtered off, washed with water (2x20 ml) and dried *in vacuo* over KOH to give 12.7 g (52.3 mmol, 95% yield) of analytically pure acid **2** as a white solid. ^{1}H NMR (DMSO-d_6): δ 7.77 (d, J=9.0 Hz, 1 H), 7.67 (d, J=16.0 Hz, 1H), 7.01 (d, J=9.0 Hz, lH), 6.51 (d, J=16.0 Hz, lH), 3.90 (s, 3H), 3.77 (s, 3H). Anal. Calcd for $C_{11}H_{11}ClO_4$: C, 54.45; H, 4.57; Cl, 14.61. Found: C, 54.20; H, 4.17; Cl, 14.66.

3-(3-Chloro-2,4-dimethoxyphenyl)propanoic acid (3). A suspension of **2** (2.57 g, 1.88 mmol) in a mixture of MeOH (50 ml) and THF (200 ml) was hydrogenated at 50 psi in the presence of 10% Pd/C (0.3 g) for 4 h. The catalyst was removed by filtration through Celite and the filtrate was concentrated to afford 12.6 g (51.5 mmol, 99% yield) of analytically pure **3** as a white solid. ^{1}H NMR (DMSO-d_6): δ 7.15 (d, J=8.6 Hz, lH), 6.86 (d, J=8.6 Hz, lH), 3.81 (s, 3H), 3.75 (s, 3H), 2.78 (t, J=7.5 Hz, 2H), 2.49 (t, J=7.5 Hz, 2H). Anal. Calcd for $C_{11}H_{13}ClO_4$: C, 54.00; H, 5.36; Cl, 14.49. Found: C, 53.71; H, 5.18; Cl, 14.47.

Methyl 3-(3-chloro-2,4-dihydroxyphenyl)propanoate (4a). A solution of **3** (10.0 g, 40.9 mmol) in a mixture of acetic acid (75 ml) and 48% aqueous hydrobromic acid (75

ml) was refluxed for 15 h. Reaction mixture was cooled and concentrated. The obtained solid material was dried by co-evaporation with toluene (3x100 ml) and dissolved in 100 ml of methanol. Hydrogen chloride was bubbled for 5 min. The resultant hot solution was allowed to cool to room temperature and then concentrated. The crude product was chromatographed on silica gel eluting with 1:4 EtOAc-hexane. Concentration of the pure product fractions afforded 9.2 g (98%) of the title compound as a pale tan oil, which slowly solidified during drying *in vacuo.* ^{1}H NMR (DMSO-d_6): δ 9.80 (s, lH), 8.95 (s, lH), 6.81 (d, J=8.4 Hz, lH), 6.38 (d, J=8.4 Hz, lH), 3.57 (s, 3H), 2.73 (t, J=7.5 Hz, 2H), 2.50 (t, J=7.5 Hz, 2H). Anal. Calcd for $C_{10}H_{11}ClO_4$: C, 52.08; H, 4.81; Cl, 15.37. Found: C, 51.95; H, 4.65; Cl, 15.28.

3,4,5,6-Tetrachloro-2-({3-chloro-2,4-dihydroxy-5-[2-(methoxycarbonyl)ethyl]phenyl}carbonyl)benzoic acid (5a). Tetrachlorophthalic anhydride (17.4 g, 60.8 mmol) was suspended in 160 ml of anhydrous 1,2-dichloroethane and stirred for 30 min. $AlC1_3$ (21.0 g, 158 mmol) was added in one portion (light exotherm) followed by addition of ester **4a** (14.0 g, 60.7 mmol) as a solution in anhydrous 1,2-dichloroethane (80 ml). The mixture was stirred for 15-20 min to give a clear solution. After being stirred at room temperature for 20 h, the reaction was concentrated and the resultant gummy residue was partitioned between ethyl acetate (500 ml) and cold 5 N HCl (500 ml). The aqueous phase was extracted with more ethyl acetate, the combined organic solution was washed with 1 N HCl (300 ml), saturated NaCl (300 ml) and dried over $MgS0_4$. Concentration of the solution gave a pale yellow solid; it was triturated with CH_2C1_2 (200 ml), cooled to 0°C and then filtered off. Washing with CH_2Cl_2 (2x50 ml) and drying *in vacuo* afforded 21.0 g of the desired benzophenone **5a** as a white solid. The mother liquor afforded additional 2.1 g of the product. Total yield was 23.1 g (74%). ^{1}H NMR (DMSO-d_6): δ 11.95 (br s, lH), 11.05 (br s, lH), 7.21 (s, lH), 3.54 (s, 3H), 2.75 (m, 2H), 2.45 (m, 2H).

2-({2,4-Dihydroxy-5-[2-(methoxycarbonyl)ethyl]phenyl}carbonyl)benzoic acid (5b). Compound **5b** was synthesized by analogy with **5a** starting from phthalic anhydride and methyl 3-(2,4-dihydroxyphenyl)propanoic acid (**4b**) (Gonzalez-Gomez, J. C.; Santana, L.; Uriarte, E. Synthesis (2003), (1), 27-29.) ^{1}H NMR (DMSO-d_6): δ 12.14 (s, 1H), 10.89 (s, 1H),8.00 (d, J=7.2 Hz, 1H), 7.62 (m, 2H), 7.39 (d, J=7.2 Hz, 1H), 6.76 (s, 1H), 6.37 (s, 1H), 3.45 (s, 3H), 2.56 (t, J=7.5 Hz, 2H), 2.38 (t, J=7.5 Hz, 2H).

4,5-Dichloro-2-({3-chloro-2,4-dihydroxy-5-[2-(methoxycarbonyl)ethyl]phenyl}carbonyl)benzoic acid (5c). Compound **5c** was synthesized using the procedure described for **5a** starting from 4,5-dichlorophthalic anhydride and **4a**. ^{1}H NMR (DMSO-d_6): δ 12.46 (s, lH), 8.16 (s, lH), 7.87 (s, lH), 6.90 (s, lH), 3.49 (s, 3H), 2.70 (t, J=7.3 Hz, 2H), 2.42 (t, J=7.3 Hz, 2H).

3-(4,5,6,7,13,16-Hexachloro-12,15-dihydroxy-1-oxospiro[3-hydroisobenzofuran-3,9'-xanthene]-11-yl)propanoic acid (7a). A suspension of **5a** (20.0 g, 39 mmol) and 4-chlororesorcinol (**6a**) (20.0 g, 138 mmol) in 200 ml of trifluoroacetic acid was heated at 70°C with stirring for 5 min to dissolve the solids. Methanesulfonic acid (10 ml) was added in one portion. After being stirred at 70°C for 2 h, the reaction was cooled and concentrated. The oily residue was triturated with water (500 ml) and extracted with ethyl acetate (300 ml). The organic phase was washed with 5% sodium bicarbonate solution to extract the product; the aqueous phase was washed with ether and then acidified with hydrochloric acid to pH of 3. The fine precipitate was collected by filtration on sintered glass funnel and washed with water. Drying *in vacuo* over P_20_5 afforded 21.6 g (90%) of the desired dye **7a** as an orange solid. ^{1}H NMR (DMSO-d_6): δ 11.20 (s, 1H), 10.16 (s, 1H), 7.37 (s, lH), 6.91 (s, lH), 6.82 (s, lH), 2.75 (m, 2H), 2.45 (t, J=7.5 Hz, 2H).

3-(12,15-Dihydroxy-1-oxospiro[3-hydroisobenzofuran-3,9'-xanthene]-11-yl)propanoic acid (7b). A solution of **5b** (4.8 g, 14 mmol) and resorcinol (**6b**) (2.3 g, 21 mmol) in 70 ml of TFA was prepared and cooled to 0^{o}C using ice/water bath. To this solution was slowly added 25 ml of methanesulfonic acid maintaining the temperature at 0-2^{o}C. The reaction was stirred at 0^{o}C for 15 h and poured onto 500 g of ice. Precipitated material was collected by filtration, re-suspended in water (500 ml) and treated with triethylamine to pH of ~10. The resultant dark-brown solution was heated to boiling point and allowed to slowly cool to room temperature. It was then acidified with concentrated hydrochloric acid to pH of ~2 and extracted with ethyl acetate (5x100 ml). The extract was washed with brine and dried over Na_2SO_4. Concentration gave a tan, viscous oil which crystallized upon trituration in water (~100 ml). Filtration and drying over P_2O_5 *in vacuo* afforded 5.1 g (90%) of **7b** as an orange solid. ^{1}H NMR (DMSO-d_6): δ 10.3 (br s, 1H), 10.2 (br s, 1H), 8.00 (d, J=7.2 Hz, 1H), 7.75 **(**m 2H), 7.23 **(**d**,** J=7.2 Hz,

lH), 6.71 (s, lH), 6.66 (s, lH), 6.51 (m, 2H), 6.44 (s, lH), 2.57 (m, 2H), 2.30 (t, J=7.5 Hz, 2H).

2-Methyl-4-chlororesorcinol (6c). To a solution of 2-methylresorcinol (40 g, 322 mmol) in 750 ml of methanol was added (45.17 g, 0.338 mol) of N-chlorosuccinimide. The mixture was stirred for 6 hours and concentrated. The resulting solid was triturated in 500 ml of a 2:1 mixture of hexane and ethyl acetate. The brown crystalline precipitate (succinimide) was filtered off and rinsed with a little extra solvent. The filtrate was concentrated to give the crude product, which was further purified on silica eluting with 4:1 hexane-ethyl acetate. Appropriate fractions were combined, and evaporated. The product was crystallized from 1:8 ether/hexanes to afford 33.1 g (65%) of the desired substituted resorcinol **6c**. ^{1}H NMR (DMSO-d_6) : δ 9.38 (s, 1H), 8.83 (s, 1H), 6.92 (d, J =8.5 Hz, 1H), 6.33 (d, J =8.5 Hz, 1H), 2.00 (s, 3H). Anal. Calcd for $C_7H_7ClO_2$*H_2O: C, 47.61; H, 5.14; Cl, 20.08. Found: C, 47.65; H 5.08; Cl 20.07.

3-(4,7,13,16-Tetrachloro-12,15-dihydroxy-14-methyl-1-oxospiro[3-hydroisobenzofuran-3,9'-xanthene]-11-yl)propanoic acid (7c). Compound **7c** was synthesized by analogy with **7b** starting from **5c** and 2-methyl-4-chlororesorcinol (**6c**). ^{1}H NMR (DMSO-d_6): δ 12.02 (br s, 1H), 10.09 (s, 1H), 9.96 (s, lH), 7.81 (s, 2H), 6.97 (s, lH), 6.75 (s, lH), 2.65 (m, 2H), 2.38 (s, 3H), 2.35 (t, J=7.5 Hz, 2H).

8,12,23,24-Tetrachloro-9-hydroxy-10-methylspiro[3,4-dihydro-2H-pyrano[3,2-b]xanthene-6,3'-3-hydroisobenzofuran]-2,19-dione (8c). Trifluoroacetic anhydride (125 ml) was added to a solution of acid **7c** (25.0 g, 40 mmol) in 125 ml of trifluoroacetic acid. After being stirred for 10 min, the reaction was concentrated and co-evaporated with CH_2Cl_2 (2x250 ml) to remove residual TFA. The resultant solid was dissolved in ethyl acetate (750 ml), washed with brine and dried over Na_2SO_4 at 6^{o}C for 20 h. Concentration afforded orange solid, which was suspended in 150 ml of 30% ethyl acetate in hexane, filtered and washed with more of the ethyl acetate/hexane mixture. Drying *in vacuo* afforded 19.3 g of lactone **8c** as a light pink solid. ^{1}H NMR (DMSO-d_6): δ 10.05 (s, lH), 8.31 (s, lH), 7.89 (s, lH), 6.94 (s, lH), 6.89 (s, lH), 2.94 (t, J=7.5 Hz, 2H), 2.82 (t, J=7.5 Hz, 2H), 2.39 (s, 3H).

8,12,22,23,24,25-Hexachloro-9-hydroxyspiro[3,4-dihydro-2H-pyrano[3,2-b]xanthene-6,3'-3-hydroisobenzofuran]-2,19-dione (8a). Compound **8a** was synthesized by analogy with **8c**. ^{1}H NMR (DMSO-d_6): δ 11.36 (s, 1 H), 7.44 (s, 1 H), 7.24 (s, lH), 6.97 (s, lH), 2.95 (m, 2H), 2.82 (m, 2H).

9-Hydroxyspiro[3,4-dihydro-2H-pyrano[3,2-b]xanthene-6,3'-3-hydroisobenzofuran]-2,19-dione (8b). Compound **8b** was synthesized by analogy with **8c**. ^{1}H NMR (DMSO-d_6): δ 10.21 (br s, lH), 8.00 (d, J=7.2 Hz, lH), 7.75 (m, 2H), 7.28 (d, J=7.2 Hz, lH), 7.12 (s, lH), 6.76 (s, lH), 6.70 (s, lH), 6.57 (s, 2H), 2.81 (t, J=7.5 Hz, 2H), 2.75 (m, 2H).

(3R,5S)-5-((Bis(4-methoxyphenyl)(phenyl)methoxy)methyl)pyrrolidin-3-ol (9). To a solution of (2S,4R)-(9H-fluoren-9-yl)methyl 2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-hydroxypyrrolidine-1-carboxylate (Hebert, N.; Davis, P.W.; DeBaets, E.L.; Acevedo, O.L. Tetrahedron Letters (1994), 35(51), 9509-9512) (11.45 g, 17.8 mmol) in 60 ml of DMF was added 60 ml of triethylamine. The mixture was heated at 80^{o}C with stirring for 1 h and concentrated. Resultant crude amine **9**, which also contained 9-methylene-9H-fluorene and traces of DMF and triethylamine, was used in the next reaction without additional purification.

Compound 10c. To a solution of **8c** (8.0 g, 14.5 mmol) in 200 ml of anhydrous DMF was added a solution of amine **9** (17.8 mmol) and triethylamine (5 ml) in 100 ml of DMF. The reaction was stirred at room temperature overnight and treated with N-methylimidazole (4.52 ml, 56.96 mmol) and trimethylacetic anhydride (11.56 ml, 56.96 mmol). After 20 h, methanol (10 ml) was added to quench excess anhydride. After another 3 h, reaction was concentrated, dissolved in ethyl acetate, washed with saturated $NaHCO_3$, brine and dried over Na_2SO_4. Solid obtained after solvent evaporation was chromatographed on silica eluting with 1:1 ethyl acetate/hexane. Concentration of the pure product fractions and drying *in vacuo* afforded 11.1 g (66%) of **10c** as an amorphous solid. HR ESI: Calcd for $C_{60}H_{57}Cl_4NO_{12}$ (M+Na^+) 1146.2533, found 1146.2522.

Compound10a. Compound **10a** was synthesized using the procedure described for **10c**. HR ESI: Calcd for $C_{59}H_{53}Cl_6NO_{12}$ (M+Na^+) 1200.1597, found 1200.1571.

Compound10b. Compound **10b** was synthesized using the procedure described for **10c**.HR ESI: Calcd for $C_{59}H_{59}NO_{12}$ ($M+Na^+$) 996.3935, found 996.3202.

Compound 11c. Four portions (2.7 mmoles each) of succinic anhydride and diisipropylethylamine were added with 1 day interval to a solution of **10c** (3.0 g, 2.66 mmol) and N-methylimidazole (0.275 ml, 3.46 mmol) in 30 ml of anhydrous DMF until no starting material was observed. Diisopropylethylamine (1.86 ml, 10.64 mmol) and pentafluorophenyl trifluoroacetate (1.828 ml, 10.64 mmol) were added to the reaction mixture. After 3 h, the reaction was concentrated and chromatographed on silica eluting with 2:1 hexane/ethyl acetate. Concentration of the pure product fractions afforded 3.45 g (93%) of PFP ester **11c** as an amorphous solid. HR ESI: Calcd for $C_{70}H_{60}Cl_4F_5NO_{15}$ ($M+Na^+$) 1412.2535, found 1412.2565.

Compound11a. Compound **11a** was synthesized using the procedure described for **11c**. HR ESI: Calcd for $C_{69}H_{56}Cl_6F_5NO_{15}$ ($M+Na^+$) 1466.1599, found 1466.1619.

Compound11b. Compound **11b** was synthesized using the procedure described for **11c**. HR ESI: Calcd for $C_{69}H_{62}F_5NO_{15}$ ($M+Na^+$) 1262.3937, found 1262.3978.

Preparation of CPG support 12c. To a solution of **11c** (0.5 mmol) in 60 mL of anhydrous DMF was added long chain aminoalkyl CPG (1000A, ~50 μmol/g amine loading) (15 g) followed by 0.5 mL N,N-diisopropylethylamine. The CPG was gently swirled for 20 h at room temperature, filtered, washed with DMF and ether and dried. To cap unreacted aminogroups and partially deprotected phenolic groups of the dye moiety, the CPG was suspended in 60 mL of pyridine and treated with 7 mL acetic anhydride and 7 mL N-methylimidazole for 2 h. The CPG was washed and dried as above. DMT loading was determined by treating a small portion of the CPG with 70% perchloric acid/MeOH (1:1, v/v) and reading A_{498} absorbance. The DMT loading of 24 μmol/g was found for the CPG.

CPG **12a** and **12b** were prepared using the procedure described for **12c**.