University of Groningen

A Fluorogenic Reaction Based on Heavy-Atom Removal for Ultrasensitive DNA Detection

Prusty, Deepak K.; Herrmann, Andreas

Published in:
Journal of the American Chemical Society

DOI:
[10.1021/ja105181v](https://doi.org/10.1021/ja105181v)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
Prusty, D. K., & Herrmann, A. (2010). A Fluorogenic Reaction Based on Heavy-Atom Removal for Ultrasensitive DNA Detection. *Journal of the American Chemical Society*, 132(35), 12197-12199.
<https://doi.org/10.1021/ja105181v>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Supporting Information

A Fluorogenic Reaction Based on Heavy-Atom Removal for Ultrasensitive DNA Detection

*Deepak K. Prusty and Andreas Herrmann**

Department of Polymer Chemistry, Zernike Institute for Advanced Materials,
University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands.
Email: a.herrmann@rug.nl

Appendices

1. Materials and Methods.....	S2
2. Synthesis and Characterization of BODIPY Probes.....	S3
3. Synthesis and Characterization of ODN Conjugates.....	S5
4. DNA-Templated Heck Reaction.....	S12
5. Reaction Conditions for Kinetic Studies.....	S14
6. Determination of Limit of Detection.....	S14

1. Materials and Methods

All chemicals and reagents were purchased from commercial suppliers and used without further purification, unless otherwise noted. The pyrrole-2-carboxaldehyde (98%), 3,5-dimethylpyrrole-2-carboxaldehyde (95%), 2,2,2-trichloroethanol (99%), esterase solution from porcine liver (PLE, 150 U/mg), N-hydroxy-succinimide (NHS, 98%), tri-*tert*-butylphosphine ($P(t\text{-Bu})_3$, 98%), tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$), sodium-tetrachloro-palladate(II) (Na_2PdCl_4 , 99.99%), 5-hexenoic acid (98%), pyridine (99%), piperidine (99%), *N,N'*-dicyclohexylmethylamine (CY_2NMe , 97%), methyl-5-hexenoate (95%) and 1,4-dioxane (99%) were purchased from Sigma-Aldrich and used as received. Other special chemicals obtained from different chemical sources were tris(3-sulfonatophenyl)phosphine hydrate sodium salt ($P(p\text{-SO}_3C_6H_4Na)_3$, Strem Chemicals), ethyl hydrogen malonate (96%, Alfa Aesar) and *N,N'*-dicyclohexyl-carbodiimide (99%, Merck). Both modified and unmodified oligonucleotides (ODNs) were synthesized using standard automated solid-phase phosphoramidite coupling methods on an ÄKTA oligopilot plus (GE Healthcare) DNA synthesizer. All solvents and reagents for oligonucleotide synthesis were purchased from Novabiochem (Merck, UK) and SAFC (Sigma-Aldrich, Netherlands). Solid supports (Primer SupportTM, 200 $\mu\text{mol/g}$) from GE Healthcare were used for the synthesis of DNA. Oligonucleotides were purified by reverse-phase High Pressure Liquid Chromatography (HPLC) using a C15 RESOURCE RPCTM 1 mL reverse phase column (GE Healthcare) through custom gradients using elution buffers (A: 100 mM triethylammonium acetate (TEAAc) and 2.5% acetonitrile, B: 100 mM TEAAc and 65% acetonitrile). Fractions were further desalted by either desalting column (HiTrapTM desalting, GE Healthcare) or dialysis membrane (MWCO 2000, Spectrum[®] Laboratories). Labeled oligonucleotides were purified by HPLC and characterized by MALDI-TOF mass spectrometry using a 3-hydroxypicolinic acid matrix. The spectra were recorded on an ABI Voyager DE-PRO MALDI TOF (delayed extraction reflector) Biospectrometry Workstation mass spectrometer. ¹H-NMR and ¹³C-NMR spectra were recorded on a Varian Mercury (400 MHz) NMR spectrometer at 25°C. High-resolution mass spectra (HRMS) were recorded on an AEI MS-902 (EI+) instrument. Absorption and fluorescence spectra of both the non-templated and templated products of the fluorogenic reactions and the concentration of the DNA were measured on a SpectraMax M2 spectrophotometer (Molecular Devices, USA) using 1 cm light-path quartz cuvette. Column chromatography was performed using silica gel 60 Å (200-400 Mesh).

2. Synthesis and Characterization of BODIPY Probes

2.1. Preparation of mono-iodinated BODIPY probe

Scheme S1. Synthetic route to the iodinated BODIPY propionic acid.

2.1.1. Synthesis of 2,2,2-trichloroethyl 3-[5-(4-Iodo-3,5-dimethylpyrrol-2-yl)methylpyrrol-2-yl]]propionate boron trifluoride adduct

The starting materials 4-iodo-3,5-dimethylpyrrole-2-carbaldehyde (**3**) and 2,2,2-trichloroethyl 3-(pyrrole-2-yl) propionate (**4**) were synthesized as reported elsewhere.^{1,2} POCl_3 (0.52 g, 3.43 mmol) in dichloromethane (5 mL) was added dropwise to a solution of compound **3** (0.57 g, 2.29 mmol) and **4** (0.83 g, 3.09 mmol) in dichloromethane (7 mL) at 0°C . The resulting reaction mixture was stirred for an additional 1 h at 0°C followed by stirring at 24°C for 4 h. Subsequently freshly distilled borontrifluoride diethyl etherate ($\text{BF}_3\cdot\text{OEt}_2$, 0.8 mL, 6.72 mmol) was added at 0°C , followed by diisopropylethylamine (2 mL, 11.4 mmol). The reaction mixture was stirred at room temperature overnight and then diluted with CH_2Cl_2 (100 mL) and washed with water (3×20 mL) and 1 M solution of citric acid (2×20 mL). The organic layer was further washed with brine (30 mL) followed by drying over Na_2SO_4 and concentration *in vacuo*. The crude product was purified by silica gel column

chromatography using hexane/EtOAc (8:2) as mobile phase yielding compound **5** as a red solid. (0.85 g, 68%).

¹H NMR (400 MHz, CDCl₃) δ (ppm): 2.23 (s, 3H), 2.62 (s, 3H), 2.92 (t, *J* = 3.3 Hz, 2H), 3.35 (t, *J* = 3.3 Hz, 2H), 4.76 (s, 2H), 6.37 (d, *J* = 7.1 Hz, 1H), 6.96 (d, *J* = 7.1 Hz, 1H), 7.15 (s, 1H).

¹³C NMR (100 MHz, CDCl₃) δ (ppm): 13.98, 16.04, 23.97, 33.05, 74.32, 82.98, 95.05, 118.12, 124.38, 129.99, 133.89, 134.35, 145.7, 158.59, 159.73, 171.03.

AEI MS-902 (EI+): Calculated exact mass for C₁₆H₁₅N₂O₂BCl₃F₂I 547.93; found 547.8.

Elemental analysis: Anal. calculated for C₁₆H₁₅N₂O₂BCl₃F₂I: C, 34.98; H, 2.75; N, 5.10; found: C, 34.87; H, 2.73; N, 5.23.

2.1.2. *Synthesis of 2,2,2-trichloroethyl 3-[5-(4-Iodo-3,5-dimethylpyrrol-2-yl)methylpyrrol-2-yl)]propionic acid boron trifluoride adduct*

To a solution of compound **5** (0.57 g, 1.03 mmol) in acetone (4 mL) and phosphate buffer (0.1 M, pH = 8.0, 15 mL) porcine liver esterase solution (PLE, 1 mL, 16.5 mg/mL, 150 U/mg) was added at 24 °C. The resulting mixture was stirred at room temperature overnight. Progress of the reaction was checked by TLC. After completion of the reaction, citric acid was added (2.0 g) and the reaction mixture was stirred for additional 15 min. The reaction mixture was diluted using ethyl acetate (50 mL). The layers were separated, with the organic layers washed with brine and dried over Na₂SO₄ and concentrated *in vacuo*. The crude product was purified by column chromatography using CH₂Cl₂/MeOH (9:1) as eluent to give compound **1** (0.273 g, 63%).

¹H NMR (400 MHz, CDCl₃) δ (ppm): 2.22 (s, 3H), 2.62 (s, 3H), 2.83 (t, *J* = 2.8 Hz, 2H), 3.3 (t, *J* = 2.8 Hz, 2H), 6.35 (d, *J* = 6.9 Hz, 1H), 6.97 (d, *J* = 6.9 Hz, 1H), 7.13 (s, 1H).

¹³C NMR (100 MHz, CDCl₃) δ (ppm): 13.68, 15.94, 24.64, 35.85, 74.05, 117.83, 123.92, 129.77, 133.41, 134.50, 145.6, 158.59, 159.72, 176.02.

AEI MS-902 (EI+): Calculated exact mass for C₁₄H₁₄N₂O₂BF₂I 418.02; found 418.88.

Elemental analysis: Anal. calculated for C₁₄H₁₄N₂O₂BF₂I: C, 40.23; H, 3.38; N, 6.7; found: C, 40.36; H, 3.42; N, 6.59.

2.2. Fluorescence activation of the mono-iodinated BODIPY probe

(c) $\text{Pd}_2(\text{dba})_3$, $\text{P}(t\text{-Bu})_3$, CY_2NMe , dioxane, RT, 1 h.

Scheme S2. Synthetic route to the fluorescent Heck coupling product.

2.2.1. Synthesis of 2,2,2-trichloroethyl 3-[5-(methyl hexenoate-3,5-dimethylpyrrol-2-yl) methylpyrrol-2-yl) propionic acid boron trifluoride adduct

$\text{Pd}_2(\text{dba})_3$ (5.6 mg, 0.006 mmol, 3 mol%) and $\text{P}(t\text{-Bu})_3$ (3.31 mg, 0.016 mmol, 4 μL , 9 mol%) were added to a solution of compound **1** (0.107 g, 0.182 mmol) in 1,4-dioxane (2 mL) and dry *N,N'*-dicyclohexylmethylamine (0.2 mL) at 24 °C followed by addition of methyl-5-hexenoate (**6**) (27.9 mg, 0.218 mmol) under argon atmosphere. The reaction mixture was stirred for 1 h at room temperature under continuous argon atmosphere. The progress of the reaction was monitored by TLC. After completion of the reaction, water (10 mL) was added to the reaction mixture. The resulting solution was extracted with CH_2Cl_2 (2 $\times$ 50 mL), followed by drying over anhydrous MgSO_4 and evaporation of the solvent under reduced pressure to obtain the crude product. Silica gel column chromatography using hexane/EtOAc (8:2) as eluent afforded compound **2** as a violet solid (53 mg, 53% yield).

^1H NMR (400 MHz, CDCl_3) δ (ppm): 1.82 (m, 2H), 2.35 (m, 2H), 2.92 (t, $J = 3.2$ Hz, 2H), 3.07 (t, $J = 3.2$ Hz, 2H), 3.32 (t, $J = 3.1$ Hz, 2H), 3.64 (s, 3H), 5.88 (m 1H), 6.21 (d, $J = 6.1$ Hz 1H), 6.34 (d, $J = 6.7$ Hz, 2H), 6.96 (d, $J = 6.7$ Hz, 2H), 7.04 (s, 1H).

^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 13.96, 16.03, 23.97, 24.68, 32.88, 34.05, 35.07, 51.15, 115.11, 118.12, 119.32, 124.38, 129.99, 133.89, 134.35, 137.47, 145.7, 158.59, 159.73, 173.03, 176.66.

HRMS (ESI⁺): Calculated exact mass for $\text{C}_{21}\text{H}_{25}\text{N}_2\text{O}_4\text{BF}_2$ 418. 19; found 418.23.

Elemental analysis: Anal. calculated for $\text{C}_{21}\text{H}_{25}\text{N}_2\text{O}_4\text{BF}_2$: C, 60.31; H, 6.02; N, 6.70; found: C, 60.39; H, 6.12; N, 5.99.

2.3. Photophysical properties of the BODIPY probe **1** and fluorescent Heck coupling product **2**

Figure S1. (A) Normalized absorption spectra of **1** (red, $\lambda_{\text{max}} = 527$) and **2** (green, $\lambda_{\text{max}} = 517$) in chloroform at 25 °C. (B) Relative fluorescence emission spectra of **1** (red, $\lambda_{\text{em}} = 536$) and **2** (green, $\lambda_{\text{em}} = 523$) in chloroform at 25 °C.

Table S1. Photophysical properties of iodinated BODIPY probe **1** and fluorescent Heck coupling product **2**.

Compound	Absorbance λ_{max} [nm]	Extinction coefficient [$\text{M}^{-1} \text{cm}^{-1}$]	Emission λ_{max} [nm]	Fluorescence quantum yield (Φ_f)
1	527	11×10^4	536	0.03
2	517	13×10^4	523	0.75

3. Synthesis and Characterization of ODN Conjugates

All oligonucleotides (**Table S2**) were synthesized on a 10 μmol scale on an ÄKTA oligopilot plus (GE Healthcare) DNA synthesizer using standard β -cyanoethylphosphoramidite coupling chemistry. Deprotection and cleavage from the PS-support was carried out by incubation in concentrated aqueous ammonium hydroxide solution for 5 h at 55 °C. Following deprotection, the oligonucleotides were purified by using anion exchange chromatography, HiTrapTM Q HP 1 mL or 5 mL column (GE Healthcare) through custom gradients using elution buffers (A: 25 mM Tris, pH = 8.0, B: 25 mM Tris and 1.0 M

NaCl). Fractions were further desalted by either desalting column (HiTrap™ desalting, GE Healthcare) or dialysis membrane (MWCO 2000, Spectrum® Laboratories). Oligonucleotide concentrations were determined by UV absorbance using extinction coefficients. Finally, the identity of the oligonucleotides was confirmed by MALDI-TOF mass spectrometry (Table S3).

Table S2. Sequences and MALDI-TOF mass spectrometry data of the probe (Iodo-BODIPY [a] and alkene-modified [b] ODNs) and template strands used for templated fluorescence activation studies using linear (L) and tritemplate (T) configurations.

Architecture	ODN No.	DNA Sequence (5' to 3')	Calculated (m/z)	Found (m/z)
L	1	NH₂ -(C6)-ATC TTT ANT TTA GCC TAG TAT ATA TCT TGC-3'	9331	9335
	2	5'-GCA AGA TAT ATA CTA GGC TAA ACT AAA GAT-(C7) NH₂	9464	9464
	3 ^a	5'-GCA AGA TAT ATA <u>G</u> TA GGC TAA ACT AAA GAT-(C7) NH₂	9504	9506
T	4	NH₂ -(C6)-TAG TAT ATA TCT TGC-3'	4736	4734
	5	5'-ATC TTT AGT TTA GC-(C7) NH₂	4453	4453
	6 ^b	5'-GCA AGA TAT ATA CTA GGC TAA ACT AAA GAT-3'	9255	9258
	7 ^c	5'-GCA AGA TAT ATA <u>G</u> TA GGC TAA ACT AAA GAT-3'	9289	9291

^aAlkene-modified oligonucleotide sequence having C to G mutation for L architecture. ^bFully matched and ^csingle-base-mismatched (C to G mutation) sequences for T architecture.

3.1. Preparation of BODIPY-labeled ODNs

Scheme S3. Synthetic route to BODIPY-labeled ODNs for DNA-templated fluorescence activation studies.

3.1.1. Synthesis of NHS ester of mono-iodo BODIPY propionic acid

The carboxyl group of mono-iodo BODIPY propionic acid was activated by reacting compound **1** (0.0229 g, 0.055 mmol) with *N*-hydroxy succinimide (NHS) (0.0182 g, 0.15 mmol) and *N,N'*-dicyclohexyl-carbodiimide (0.0185 g, 0.16 mmol) in 1 mL of DMF. The reaction was carried out for 24 h under inert atmosphere at room temperature (**Scheme S3**). Precipitated dicyclohexylurea (DCU) was removed by filtration. The solvent was evaporated under reduced pressure and the crude mixture was purified by column chromatography using hexane/EtOAc (1:1) as eluent. Activated product **7** was obtained as violet solid (16 mg, 57%).

3.1.2. DNA labeling with mono-iodo BODIPY NHS ester

5'-(C6) Amino-modified oligonucleotides ODN1 and 4 used in L- and T-architectures respectively, (**Table S2**) were dissolved in sodium tetraborate buffer (0.1 M, pH = 8.5) in two separate vials at concentrations of 1 nmol/μL. 100 μL of each amino-modified oligonucleotide solution was reacted separately in two different vials, each containing a solution of activated BODIPY NHS ester **7** in dimethylsulfoxide (20 μL, 40 μg/μL). The resulting reaction

mixtures were mixed in a shaker for 24 h at ambient temperature (**Scheme S3**). The reaction mixtures were freeze-dried to remove the DMSO-H₂O mixture. Purification of the labeled oligonucleotides was carried out by using reverse-phase HPLC on a C15 RESOURCE RPCTM 1 mL column (GE Healthcare) through custom gradients using elution buffers (A: 100 mM TEAAc and 2.5% acetonitrile, B: 100 mM TEAAc and 65% acetonitrile). The coupling yield of the labeling reaction was estimated to be 40% from the integration of the peaks of the HPLC chromatogram. The purified BODIPY-labeled oligonucleotides (band at ~7 min of **Figure S2A**) were analyzed by UV-Vis absorption spectroscopy (**Figure S2B**) and MALDI-TOF mass spectrometry (**Figure S3**).

Figure S2. (A) Reverse phase HPLC chromatogram of BODIPY-ODN4 crude mixture. Elution is monitored at 260 nm. Band eluted at ~7 min was fractionated and analyzed by UV/Vis spectroscopy. (B) Absorption spectra of the fractionated BODIPY-ODN4. Peaks at 260 and 527 nm correspond to ODN and BODIPY absorbance respectively.

Figure S3. MALDI-TOF mass spectra of BODIPY-labeled ODNs (A) BODIPY-ODN1: calculated 9732; found 9737. (B) BODIPY-ODN4: calculated 5137; found 5138.

3.2. Conjugation of 5-hexenoic acid with amino modified ODNs

Scheme S4. Synthetic route to the alkene-modified ODNs.

3.2.1. Synthesis of NHS ester of 5-hexenoic acid

N-hydroxysuccinimide (0.786 g, 6.83 mmol) and dicyclohexylcarbodiimide (1.87 g, 9.11 mmol) were added to a solution of 5-hexenoic acid **8** (0.524 g, 4.59 mmol) in dry DMF (4 mL), and stirred under argon for 24 h (**Scheme S4**). After completion of the reaction precipitated DCU was removed by filtration and DMF was evaporated under reduced pressure. The crude product was purified by column chromatography using dichloromethane as eluent. NHS activated product **9** was obtained as a colorless viscous oil (0.75 g, 78% yield).

3.2.2. Conjugation of amino- modified ODNs with 5-hexenoic acid NHS ester

3'-(C7) Amino-modified oligonucleotides ODN2, ODN3 and ODN5 (**Table S1**) were dissolved in sodium tetraborate buffer (0.1 M, pH = 8.5) in three separate vials at concentrations of 1 nmol/μL. 100 μL (100 nmol) of each amino modified oligonucleotide solution was mixed with 20 μL solution of activated NHS ester **9** (20 μg/μL) in dimethylsulfoxide. The vials were shaken for 24 h at ambient temperature (**Scheme S4**). The reaction mixtures were freeze-dried to remove the DMSO-H₂O. Purifications of the alkene-labeled oligonucleotides were carried out using reverse-phase High Pressure Liquid Chromatography (HPLC) on a C15 RESOURCE RPCTM 1 mL column (GE Healthcare) through custom gradients using elution buffers (A: 100 mM TEAAc and 2.5% acetonitrile, B: 100 mM TEAAc and 65% acetonitrile). The purified alkene-modified oligonucleotide strands were analyzed by MALDI-TOF mass spectrometry. Alkene-ODN2: 38% yield, MALDI-TOF mass calculated 9561; found 9563. Alkene-ODN3: 43% yield, MALDI-TOF mass calculated 9600; found 9600. Alkene-ODN5: 49% yield, MALDI-TOF mass calculated 4550; found 4552.

4. DNA-Templated Heck Reaction

4.1. Reaction conditions for templated fluorogenic reactions

Both the L- and T-type DNA-templated C-C coupling reactions were performed using probe and template concentrations of 120 nM in the presence of water-soluble Pd catalyst (200 μM Na_2PdCl_4 and 400 μM $\text{P}(p\text{-SO}_3\text{C}_6\text{H}_4\text{Na})_3$) in NaOAc buffer (0.5 M, pH = 5.0) at 24 °C. DNA strands and buffer were mixed for 15 min in the presence of 75 mM NaCl solution, followed by the addition of catalyst stock solution (5 mM in water) to initiate the reaction. The reaction mixtures, each with a final volume of 150 μL , were shaken for 4 h at 24 °C. Reaction mixtures were then desalted by gel filtration using Sephadex G-25 and purified by anion exchange chromatography, HiTrapTM Q HP 1 mL column (GE Healthcare) through custom gradients using elution buffers (A: 25 mM Tris, B: 25 mM Tris and 1.0 M NaCl). The collected product was further desalted using Sephadex desalting column (HiTrapTM desalting, 5 mL, GE Healthcare). Finally, the purified templated products were analyzed by MALDI-TOF mass spectrometry (Figure S4 and S5).

Figure S4. AIEX HPLC analysis of T-templated reaction mixture. Peak A (~18 min) and B (~22 min) correspond to unreacted Alkene-ODN5 and BODIPY-ODN4 conjugates, respectively, whereas peak C at ~37 min is the ODN4-BODIPY-ODN5 ligation product.

Figure S5. MALDI-TOF mass spectrum of L-templated (ODN1-BODIPY-ODN2) ligation product: calculated 19121; found 19118.

Figure S6. MALDI-TOF mass spectrum of T-templated (ODN4-BODIPY-ODN5) ligation product: calculated 9557; found 9559.

5. Reaction Conditions for Kinetic Studies

The kinetic experiments of fluorogenic heavy-atom displacement in T architecture was carried out under the following reaction conditions: pH = 5.0, 24 °C, 50 μ M Na₂PdCl₄ and 100 μ M P(*p*-SO₃C₆H₄Na)₃, 75 mM NaCl, and 30 nM of probe, reagent and template ODNs. The fluorescence data were recorded on a SpectraMax M2 spectrophotometer (Molecular Devices, USA) using a 1 cm light-path quartz cuvette. The reaction fluorescence was monitored every 30 sec for 1 h at 523 nm (excitation: 515 nm).

6. Determination of Limit of Detection

The detection limit of the DNA-templated fluorogenic conversion was calculated by a reported method.³ We carried out a number of T-templated fluorogenic reactions using equimolar concentrations of probe, reagent and template ODNs ranging from 1 nM to 1 pM. As a negative control, all reactions were also performed without template. The fluorescence intensity after reaction completion was measured for all reaction and the resulting fluorescence intensities were plotted against template concentration. The limit of detection was determined to be the lowest measured concentration for which the mean fluorescence intensity exceeded that of the negative control by at least three standard deviations, i.e. I_0 (0.001 nM) = 9.211 ± 0.69 ; I_0 (0.001 nM) + $3 \times \text{sd}$ = $11.28 < 12.96 \pm 0.87 = I_0$ (0.01 nM). Thus 10 pM was determined to be the detection limit.

References

1. Wan, C. W.; Burghart, A.; Chen, J.; Bergstrom, F.; Johansson, L. B. A.; Wolford, M. F.; Kim, T. G.; Topp, M. R.; Hochstrasser, R. M.; Burgess, K. *Chem. Eur. J.* **2003**, 9, (18), 4430-4441.
2. Malan, S. F.; van Marle, A.; Menge, W. M.; Zuliana, V.; Hoffman, M.; Timmerman, H.; Leurs, R. *Bioorg. Med. Chem.* **2004**, 12, (24), 6495-6503.
3. Baeumner, A. J.; Pretz, J.; Fang, S. *Anal. Chem.* **2004**, 76, 888-849.