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Supporting Information

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Four color, enzyme-free interrogation of DNA sequences with chemically activated, 3'-fluorophore-labeled nucleotides

by

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1. General and Abbreviations

General. Anhydrous solvents were purchased over molecular sieves and used without further purification. Reagents were from Acros (Geel, Belgium) or Aldrich/Fluka/Sigma (Deisenhofen, Germany). 2'-Deoxyadenosine 5'-monophosphate (5'-AMP) and 2'-deoxycytidine 5'-monophosphate (5'-CMP) were from Acros (Geel, Belgium). 2'-Deoxyguanosine 5'-monophosphate (5'-GMP), thymidine 5'-monophosphate (5'-TMP), and EDC (*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide) were from Fluka (Deisenhofen, Germany). 7-Aza-1-hydroxybenzotriazole (HOAt) was from MoBiTec (Göttingen, Germany). Dowex 50 WX8-200 cation exchange resin was from Acros (Geel, Belgium). Samples of 3'-azido-3'-deoxy-thymidine (AZT) were purchased from Toronto Research Chemicals Inc. (Ontario, Canada) or synthesized in-house according to a literature procedure.[1] The phosphoramidite building blocks of Cy3, Cy3.5, Cy5, and Cy5.5 were obtained from Amersham (Freiburg, Germany). Unmodified DNA oligomers and Cy-labeled DNA oligomers for calibration experiments were purchased from VBC-GENOMICS (Vienna, Austria) in HPLC-purified form and were used without further purification. Reversed phosphoramidites for dA^{Bz}, dC^{Bz}, dG^{iBu}, and dT, DMT-protected hexaethylenglycol phosphoramidite and the phosphorylation reagent for the 5'-hydroxyl groups were from Chemgenes (Ashland, MA, USA). Other reagents for DNA synthesis were from Proligo (Hamburg, Germany). Aldehyde-coated glass slides were purchased from Genetix Ltd. (New Milton, UK). Buffers and solutions for microarray experiments (PBS buffer, pH 7.0; 20-fold concentrated SSC solution, pH 7.4; and MES buffer pH 6.3) were prepared following standard recipes.[2] Labeled nucleotides **7a-t** were quantified using the following extinction coefficients for the dye residues: Cy3 λ_{max} 541 nm ε = 150000 M^{-1} cm^{-1}, Cy3.5 λ_{max} 586 nm ε = 116000 M^{-1} cm^{-1}, Cy5 λ_{max} 646 nm ε = 250000 M^{-1} cm^{-1}, Cy5.5 λ_{max} 688 nm ε = 209000 M^{-1} cm^{-1}.

NMR spectra were recorded on Bruker AC250 or AVANCE 500 spectrometers. MALDI-TOF mass spectra were acquired on a Bruker REFLEX IV spectrometer, using software packages XACQ 4.0.4 and XTof 5.1. Oligonucleotides were measured in negative, linear mode and monomers in positive, linear mode. MALDI matrices were applied as a mixture of 2,4,6-trihydroxyacetophenone (0.3 M in EtOH) and diammonium citrate (0.1 M in H_2O) at a ratio of 2:1 (v/v). UV-Vis spectra were recorded on a Perkin Elmer spectrophotometer Lambda 10 and fluorescence spectra were recorded on a Perkin Elmer luminescence spectrometer LS50B. Microarrays were scanned using an ArrayWoRx® biochip reader (Applied Precision, Issaquah, USA).

Abbreviations. DIEA, diisopropylethylamine; DMAP, 4-(*N*,*N*-dimethylamino)-pyridine; HOAt, 7-aza-1-hydroxybenzotriazole; dAMP, 2'-deoxyadenosine 5'-monophosphate; dCMP, 2'-deoxycytidine 5'-monophosphate; dGMP, 2'-deoxyguanosine 5'-monophosphate; dTMP, thymidine 5'-monophosphate; EDC, *N*-(3-dimethylaminopropyl)-*N*'-ethyl-carbodiimide; THAP, 2,4,6-trihydroxyacetophenone; r.t., room temperature.

2. Synthesis of Labeled Nucleotides

Solid-phase synthesis of 3'-labeled 5'-monophosphates (7a-t) (General Protocol 1).

The synthesis of labeled 5'-monophosphates **7a-t** was performed on a 8909 Expedite DNA synthesizer (Perseptive Biosystems), using phosphoramidite chemistry. Each synthesis was run on a 2 µmol scale in duplicate, starting from dA^{Bz}-loaded controlled pore glass (loading 33 µmol/g). As documented below, the coupling time for (reversed) phosphoramidites was extended. The concentration of the Cy phosphoramidites was lowered to 0.05 M, all other phosphoramidites were used at the usual concentration of 0.1 M in CH_3CN. Figure S1, below, lists the steps for a coupling cycle.

```
/* ------------------------------------------------------------------------------- */
/*       Function              Mode  Amount Time(sec)        Description           */
/*                                   /Arg1   /Arg2                                 */
/* ------------------------------------------------------------------------------- */
$Deblocking
 144 /*Index Fract. Coll.  */ NA         1     0  "Event out ON"
   0 /*Default             */ WAIT       0   1.5  "Wait"
 141 /*Trityl Mon. On/Off  */ NA         1     1  "START data collection"
  16 /*Dblk                */ PULSE     20     0  "Dblk to column"
  16 /*Dblk                */ PULSE    100    75  "Deblock"
  38 /*Diverted Wsh A      */ PULSE     60     0  "Flush system with Wsh A"
 141 /*Trityl Mon. On/Off  */ NA         0     1  "STOP data collection"
  38 /*Diverted Wsh A      */ PULSE     60     0  "Flush system with Wsh A"
 144 /*Index Fract. Coll.  */ NA         2     0  "Event out OFF"
$Coupling
   1 /*Wsh                 */ PULSE      5     0  "Flush system with Wsh"
   2 /*Act                 */ PULSE     15     3  "Flush system with Act"
  18 /*A + Act             */ PULSE      7     0  "Monomer + Act to column"
   0 /*Default             */ WAIT       0   300  "Default"
  18 /*A + Act             */ PULSE      8    20  "Couple monomer"
   0 /*Default             */ WAIT       0   300  "Default"
  18 /*A + Act             */ PULSE      7    20  "Couple monomer"
   0 /*Default             */ WAIT       0   300  "Default"
   2 /*Act                 */ PULSE      8    50  "Couple monomer"
   1 /*Wsh                 */ PULSE     10    56  "Couple monomer"
   1 /*Wsh                 */ PULSE      8     0  "Flush system with Wsh"
$Capping
  12 /*Wsh A               */ PULSE     20     0  "Flush system with Wsh A"
  13 /*Caps                */ PULSE     20     0  "Caps to column"
  12 /*Wsh A               */ PULSE      6    15  "Cap"
  12 /*Wsh A               */ PULSE     14     0  "Flush system with Wsh A"
$Oxidizing
  15 /*Ox                  */ PULSE     20     0  "Ox to column"
  12 /*Wsh A               */ PULSE     15     0  "Flush system with Wsh A"
$Capping
  13 /*Caps                */ PULSE      7     0  "Caps to column"
  12 /*Wsh A               */ PULSE     30     0  "End of cycle wash"
```

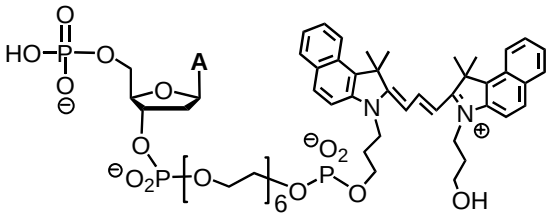
Figure S1. Coupling cycle for the synthesis of nucleotides **7a-t**.

Deprotection from the solid support and purification on RP-phase (General Protocol 2).

All labeled 5'-monophosphates were deprotected and cleaved from the solid support using conc. aqueous ammonia (25 %) (1 mL) for 24 h at r.t. The supernatant was collected and the residue was washed with deionized water. Excess ammonia was removed from the combined aqueous solutions with a gentle stream of compressed air directed onto the surface of the solutions. The 5'-monophosphates were purified on a Sep-Pak® Vac 3cc (C18) cartridge (Waters Corporation, Milford, Massachusetts) using a step gradient (in 2.5 % steps) of CH_3CN (solvent B) in 0.1 M triethylammonium acetate buffer, pH 7 (solvent A). The yield was

calculated by UV-Vis measurements using the loading of the support and the molar extinction coefficient of the Cy-dyes.

P-dA-Cy3.5 (7a)



Compound **7a** was prepared using General Protocols 1 and 2. The product eluted between 35 and 55 percent of CH_3CN from the Sep-Pak cartridge. Yield: 2.56 mg, 2 µmol, 50 %.

^{1}H-NMR: (500 MHz, D_2O): δ 8.21(s, 1H), 8.15 (t, *J*=12.9 Hz, 1H), 7.84 (s, 1H), 7.76-7.59 (m, 6H), 7.33-7.188 (m, 6H), 6.15 (d, *J*=12.9 Hz, 2H), 6.07 (m, 1H), 5.37 (m, 1H), 4.86 (m, 1H), 4.28 (m, 1H), 4.08-3.94 (m, 4H), 3.89 (m, 4H), 3.78 (m, 4H), 3.56 (t, *J*=6.5 Hz, 2H), 3.53 (t, *J*=6.5 Hz, 2H), 3.47-3.24 (m, 18H), 2.53-2.46 (m, 2H), 1.87 (t, *J*=6.5 Hz, 2H), 1.76 (t, *J*=6.5 Hz, 2H), 1.47 (s, 12H).

^{13}C-NMR: (125 MHz, D_2O): δ 182.5, 177.7, 175.4, 152.1, 151.5, 149.1, 148.3, 138.9, 138.9, 132.8, 127.1, 127.0, 125.2, 124.9, 110.8, 101.8, 85.2, 83.7, 83.6, 83.4, 70.1, 70.0, 69.5, 69.4, 69.3, 64.8, 58.4, 53.9, 50.4, 29.6, 26.9, 26.8.

^{31}P-NMR: (202 MHz, D_2O): δ 0.65, 0.29, -0.36.

MALDI-TOF MS (linear, positive): calcd for $C_{59}H_{79}N_7O_{19}P_3$ ($[M+H]^+$): 1283.2, found 1283.5.

HPLC: CH_3CN gradient: 0% for 5 min to 43 % in 42 min, t_R = 52.8 min.

UV-Vis: λ_{max} (relative intensity); 585 (75 %), 260 (8 %).

Fluorescence: λ_{max} = 620 nm.

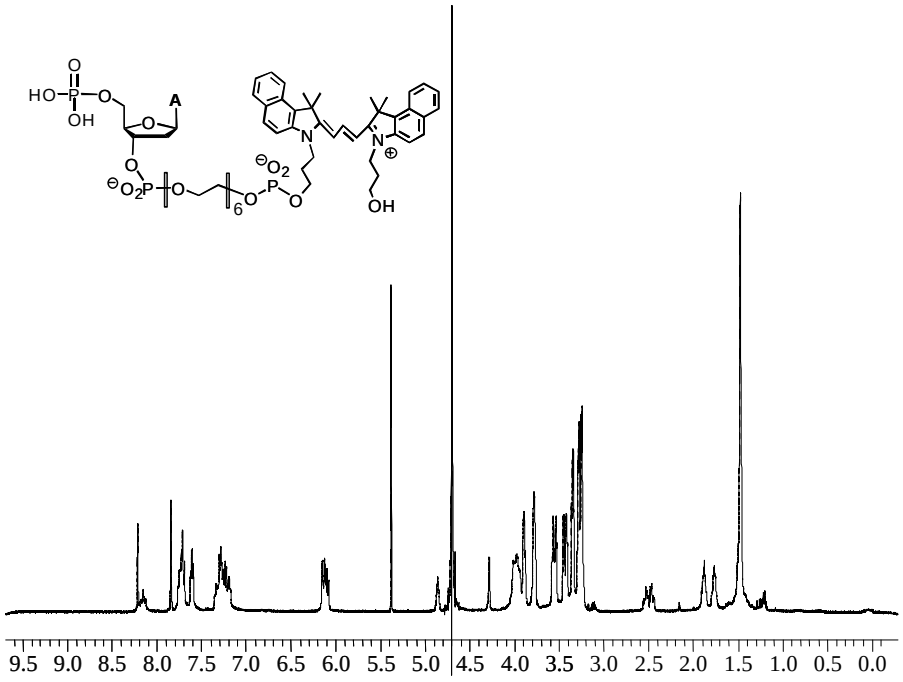
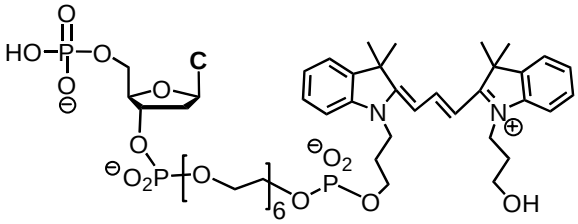


Figure S2. **^{1}H-NMR** (500 MHz, D_2O) of compound **7a**.

P-dC-Cy3 (7c)



Prepared using General Protocols 1 and 2. The product eluted between 30 and 50 % of acetonitrile from the Sep-Pak cartridge. Yield: 2.6 mg, 2.24 µmol, 56%.

^{1}H-NMR: (500 MHz, D_2O): δ 8.41 (q, *J*=13.2 Hz, 1H), 7.85 (d, *J*=7.5 Hz, 1H), 7.47-7.23 (m, 8 H), 6.30 (d, *J*=13.8 Hz, 1H), 6.29 (d, *J*=13.8 Hz, 1H), 6.25 (dd, *J*= 2.6, 8.3 Hz, 1H), 5.98 (d, *J*=7.5 Hz, 1H), 4.75 (m, 1H), 4.25 (m, 1H), 4.14 (t, *J*=6.5 Hz, 2H), 4.10 (t, *J*=6.5 Hz, 2H), 3.95 (m, 2H), 3.89 (m, 2H), 3.84 (m, 4H), 3.63-3.45 (m, 24H), 2.42 (m, 1H), 2.15 (m, 1H), 2.06 (t, *J*=6.8 Hz, 2H), 1.95 (t, *J*=6.8 Hz, 2H), 1.62 (s, 12H).

^{13}C-NMR: (125 MHz, D_2O): δ 175.0, 174.9, 165.5, 156.7, 150.9, 141.9, 141.8, 141.7, 141.0, 128.8, 128.7, 125.4, 122.3, 111.3, 111.1, 102.3, 96.4, 85.9, 84.9, 84.8, 84.7, 76.1, 76.0, 70.1, 70.0, 69.9, 69.6, 69.5, 64.8, 64.7, 64.6, 64.5, 62.5, 62.4, 58.6, 49.2, 41.0, 40.6, 38.8, 29.2, 27.9, 27.8, 27.3, 27.1.

^{31}P-NMR: (202 MHz, D_2O): δ 0.67, 3.37, -0.38.

MALDI-TOF MS (linear, positive): calcd for $C_{50}H_{75}N_5O_{20}P_3$ ($[M+H]^+$): 1159.1, found 1159.0.

HPLC: CH_3CN gradient: 0% for 5 min to 43% in 42 min, t_R = 38.1 min.

UV-Vis: λ_{max} (relative intensity); 545 (60 %), 260 (13 %).

Fluorescence: λ_{max} = 574 nm.

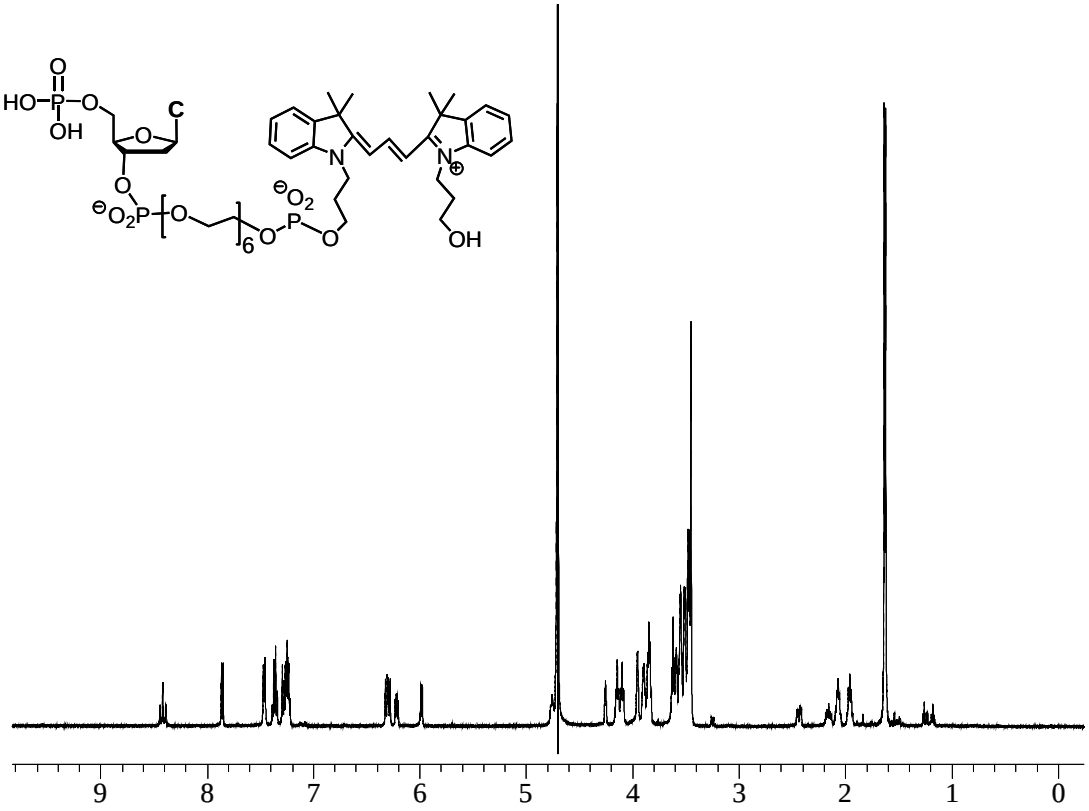
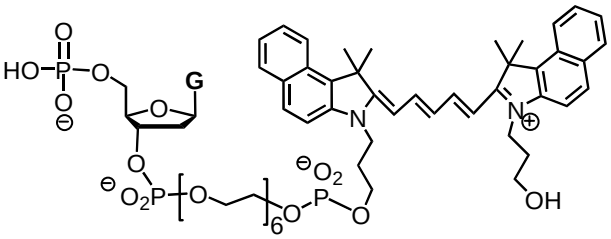


Figure S3. **^{1}H-NMR** (500 MHz, D_2O) of compound **7c**.

P-dG-Cy5.5 (7g)



Prepared using General Protocols 1 and 2. The product eluted between 40 and 60 percent of acetonitrile from the Sep-Pak cartridge. Yield: 0.9 mg, 0.68 µmol, 17%.

^{1}H-NMR: (500 MHz, D_2O): δ 7.8-7.41 (m, 12H), 7.32 (m, 1H), 7.20 (m, 1H), 7.01 (m, 1H), 6.31 (m, 1H), 6.08 (m, 1H), 5.99 (m, 1H), 5.90 (m, 1H), 4.85 (m, 1H), 4.25 (m, 1H), 4.17-4.15 (m, 4H), 3.88-

3.79 (m, 8H), 3.67 (m, 2H), 3.53 (m, 2H), 3.43 (m, 2H), 3.39-3.25 (m, 18H), 2.53-2.35 (m, 2H), 2.04 (m, 2H), 1.95 (m, 2H), 1.51 (m, 6H), 1.41 (m, 6H).

^{31}P-NMR: (202 MHz, D_2O): δ 1.26, 0.70, -0.36.

MALDI-TOF MS (linear, positive): calcd for $C_{61}H_{81}N_7O_{20}P_3$ ($[M+H]^+$): 1325.2, found 1325.5.

HPLC: CH_3CN gradient: 0% for 5 min to 43% in 42 min, t_R = 50.9 min.

UV-Vis: λ_{max} (relative intensity); 680 (61 %), 260 (15 %).

Fluorescence: λ_{max} = 716 nm.

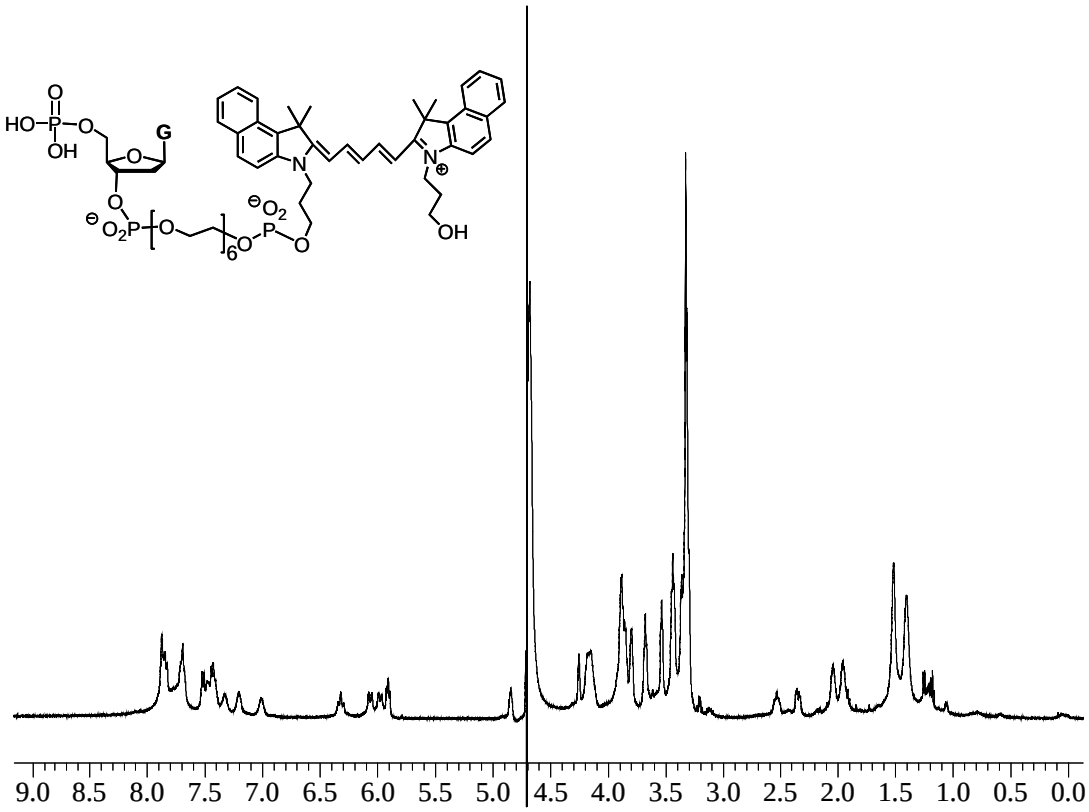
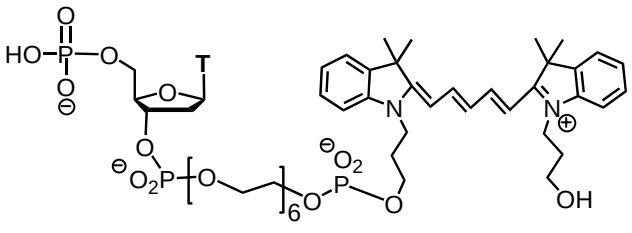


Figure S4 ^{1}H-NMR (500 MHz, D_2O) of compound **7g**.

P-dT-Cy5 (7t)



Prepared using General Protocols 1 and 2. Elution from Sep-Pak cartridge between 30 and 50% acetonitrile. Yield: 1.9 mg, 1.6 µmol, 40%.

^{1}H-NMR: (500 MHz, D_2O): δ 7.86 (m, 2H), 7.70 (s, 1H), 7.41-7.11 (m, 8H), 6.44 (t, *J*=12.8 Hz, 1H), 6.23 (dd, *J*= 2.9, 5.8 Hz, 1H), 6.16 (d, *J*=13.8 Hz, 2H), 4.78 (m, 1H), 4.23 (m, 1H), 4.12 (t, *J*=6.5 Hz, 2H), 4.08 (t, *J*=6.5 Hz, 2H), 3.92 (m, 2H), 3.89 (m, 2H), 3.84 (m, 4H), 3.62-3.47 (m, 24H), 2.35-2.25 (m, 2H), 2.04 (t, *J*=7.3 Hz, 2H), 1.94 (t, *J*=7.3 Hz, 2H), 1.79 (s, 3H), 1.57 (s, 12H).

^{13}C-NMR: (125 MHz, D_2O): δ 173.3, 166.4, 153.4, 151.5, 142.0, 141.9, 141.3, 137.5, 128.5, 128.4, 124.9, 124.7, 122.2, 111.7, 110.9, 110.8, 103.1, 103.0, 85.1, 85.0, 84.9, 76.4, 76.3, 70.1, 70.0, 69.9, 69.6, 69.5, 69.4, 64.8, 64.7, 64.6, 64.5, 64.3, 64.2, 62.6, 62.5, 58.9, 58.6, 49.0, 40.8, 40.4, 37.9, 29.3, 27.9, 26.9, 26.8, 11.6, 7.4.

^{31}P-NMR: (202 MHz, D_2O): δ 1.38, 0.66, -0.40.

MALDI-TOF MS (linear, positive): calcd for $C_{53}H_{78}N_4O_{21}P_3$ ($[M+H]^+$): 1200.1, found 1200.5.

HPLC: CH_3CN gradient: 0% for 5 min to 43 % in 42 min, t_R = 42.7 min.

UV-Vis: λ_{max} (relative intensity); 532 (38 %), 260 (10 %).

Fluorescence: λ_{max} = 676 nm.

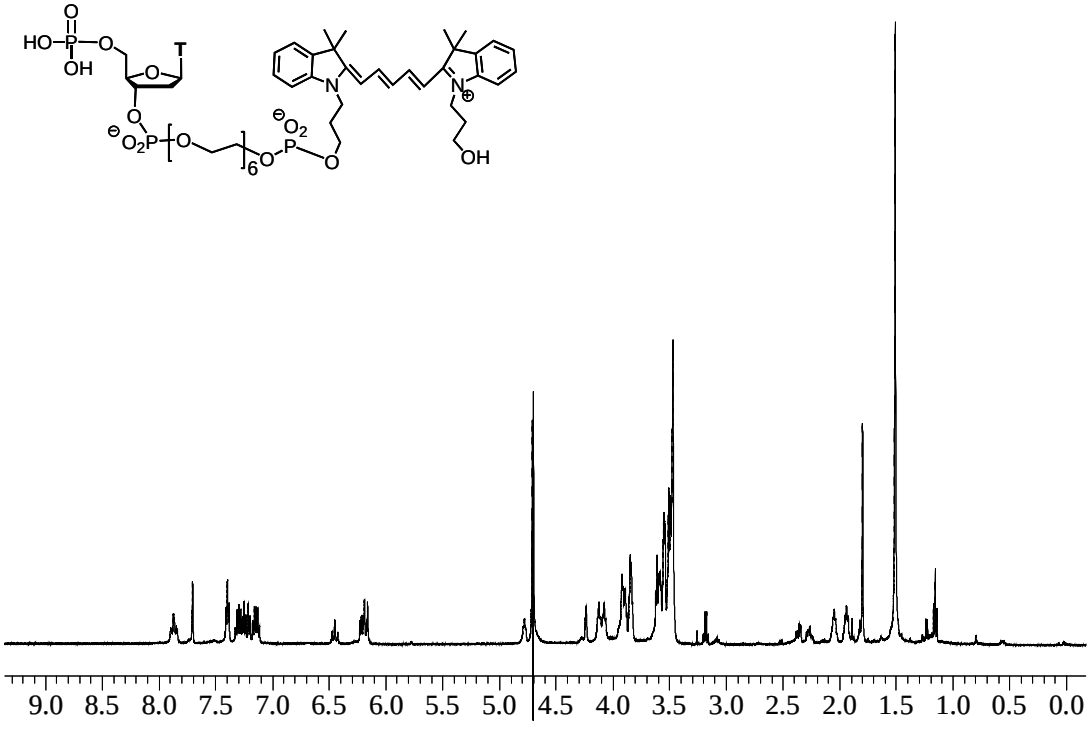


Figure S5. ^{1}H-NMR (500 MHz, D_2O) of compound **7t**.

Activation of labeled nucleotides to OAt esters 1a-t (General Protocol 3).

EDC hydrochloride (28.5 mg, 150 µmol) and HOAt (12.2 mg, 90 µmol) were weighted into a polypropylene vessel and suspended in water (100 µL). The pH was adjusted to 5.0 with NaOH solution (1.5 M; 5 µL), resulting in a clear yellow solution. After dilution of this stock solution with water (100 µL) an aliquot (0.66 µL) was added to the respective lyophilized

labeled nucleotide **(7a**, **c**, **g**, or **t)** (0.1 µmol) in a polypropylene vessel. The dark solution was left standing at r.t. for 2 h protected from light, and was then used directly for primer extension reactions.

OAt-P-dA-Cy3.5 (1a)

MALDI-TOF MS (linear, positive): calcd for $C_{64}H_{81}N_{11}O_{19}P_3$ ($[M+H]^+$): 1401.3, found 1401.5.

OAt-P-dC-Cy3 (1c)

MALDI-TOF MS (linear, positive): calcd for $C_{55}H_{77}N_9O_{20}P_3$ ($[M+H]^+$): 1277.2, found 1277.9.

OAt-P-dG-Cy5.5 (1g)

MALDI-TOF MS (linear, positive): calcd for $C_{66}H_{83}N_{11}O_{20}P_3$ ($[M+H]^+$): 1443.3, found 1443.5.

OAt-P-dT-Cy5 (1t)

MALDI-TOF MS (linear, positive): calcd for $C_{58}H_{80}N_8O_{21}P_3$ ($[M+H]^+$): 1318.2, found 1320.1.

Other Modified Nucleic Acids

Primer 8. The synthesis of 3'-amino-terminated primer **8** was performed as published earlier.[3]

Capture oligonucleotide 5'-Lys-DP-TAAAAGATACCATCAA-3' (12)

Compound **12** was prepared using a protocol published for similar capture oligonucleotides for immobilization on aldehyde-derivatized microarray sildes.[6]

3. Primer Extension Assays

Chemical Primer Extension Reaction (General Protocol 4).

Non-enzymatic, template-directed primer extension reactions were carried out in 2.5 µL of an aqueous buffer solution at r.t. The oligonucleotides template (**9a-t**), primer (**8**) and helper (**10**) were dissolved separately in water to give stock solutions (360 µM). To a stock solution of the buffer (1 µL) containing HEPES (0.5 M), NaCl (1 M) und $MgCl_2$ (0.2 M) at pH 8.9, an aliquot (0.25 µL) of each of the stock solutions of template (**9a-t**), primer (**8**) and helper (**10**) was added. To this solution were added water (0.25 µL), and 0.5 µL of a stock solution containing equal amounts of the OAt-activated monomers (either compounds **1a-t**, or unlabeled OAt esters of dAMP, dCMP, dGMP (**16a-g**), and dTMP[3] for kinetic studies). The final concentrations of the oligonucleotides was 36 µM, that of the monomers was 3.6 mM, and those of the buffer components were HEPES (0.2 M), NaCl (0.4 M) and $MgCl_2$ (0.08 M).

MALDI TOF Analysis (General Protocol 5).

MALDI-TOF mass spectrometric analysis was similar to that described earlier.[4] The mass spectra were acquired under conditions that allow for quantitative detection.[5] Briefly, aliquots from the assay solution (0.4 µL) were drawn at stated intervals and diluted with water (10 µL for unlabeled nucleotides or 20 µL for labeled nucleotides). To the solution were added a few grains of Dowex cation exchange resin (ammonium form) and the resulting mixture was left standing for 2 min. To suppress gas phase oligomers of labeled nucleotides in mass spectra, the solution was applied to MicroSpin™ G-25 columns (Amersham, Freiburg, Germany) prior to spotting. Aliquots (0.5 µL) were then spotted on a MALDI target, allowed to dry and treated with the matrix/comatrix solution (solution of THAP and diammonium citrate in ethanol and water, 2:1, typically 0.5-1 µL). Three spectra were acquired per time point, using 300 shots per spot. The intensities of the peaks were determined based on peak heights.

Table S1 Results from kinetic study on primer extension reactions with the sequence motif given in Scheme 2 at 36 µM template/primer/helper, 3.6 mM activated unlabeled monomers [a]

Oligomers primer/template/helper	k′ [$h^{-1}M^{-1}$][b]	product ratio (primer+C/T/A/G)[c]	$t_{1/2}$ [h]
8/9a/10	76	7 / **79** / 13 / <1	2.53
8/9c/10	1531	<1 / <1 / <1 / **97**	0.13
8/9g/10	661	**96** / <1 / <1 / 2	0.29
8/9t/10	205	<1 / <1 / **94** / 4	0.94

[a] aqueous buffer: 200 mM HEPES, 400 mM NaCl, 80 mM $MgCl_2$, pH 8.9.
[b] k' = second order rate constant; oligonucleotide complexes treated as one reactant, activated nucleotide as the other.
[c] Perfectly matched primer extension products are highlighted in boldface.

Plots of kinetics for primer extension with the sequence motif of Scheme 2

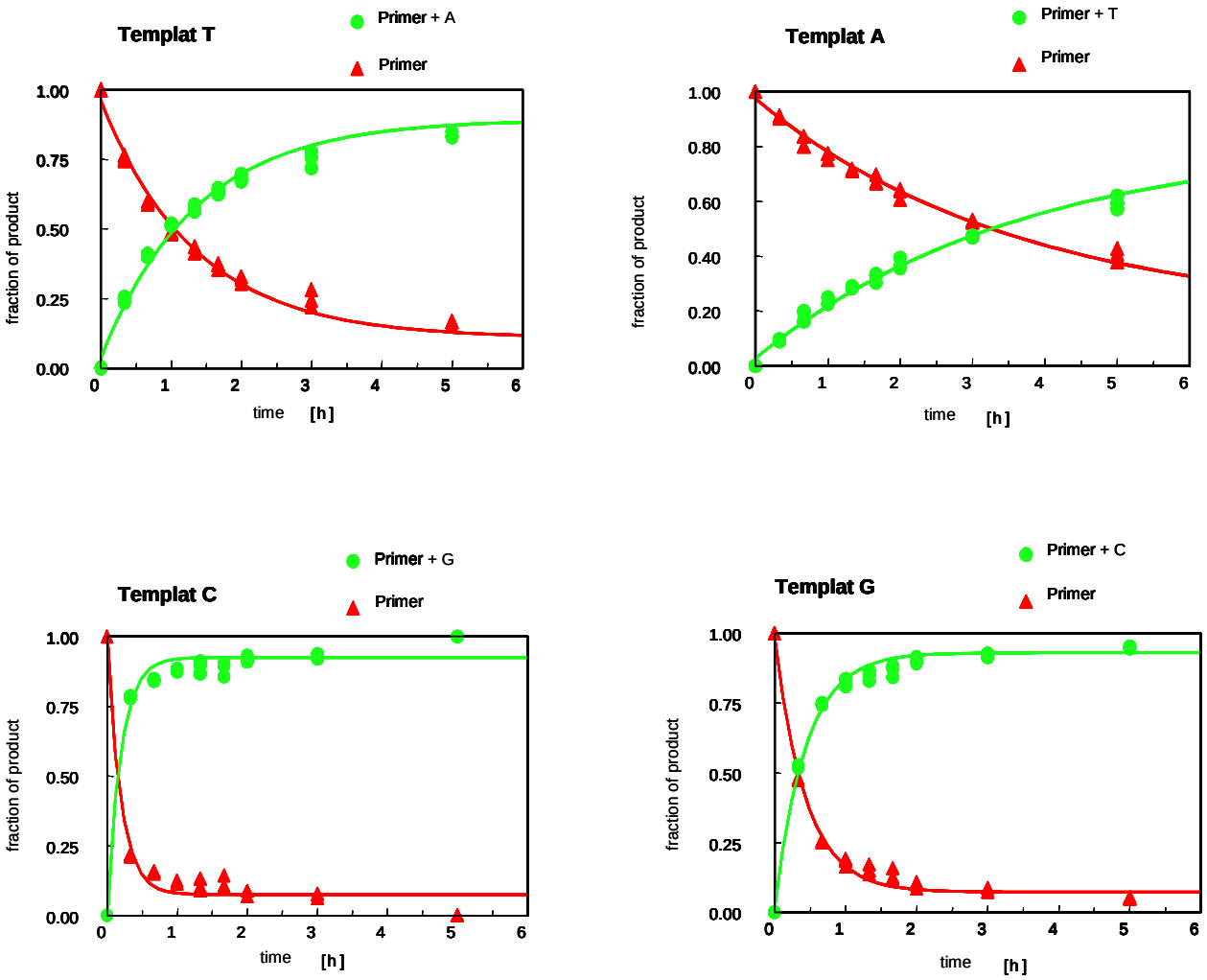
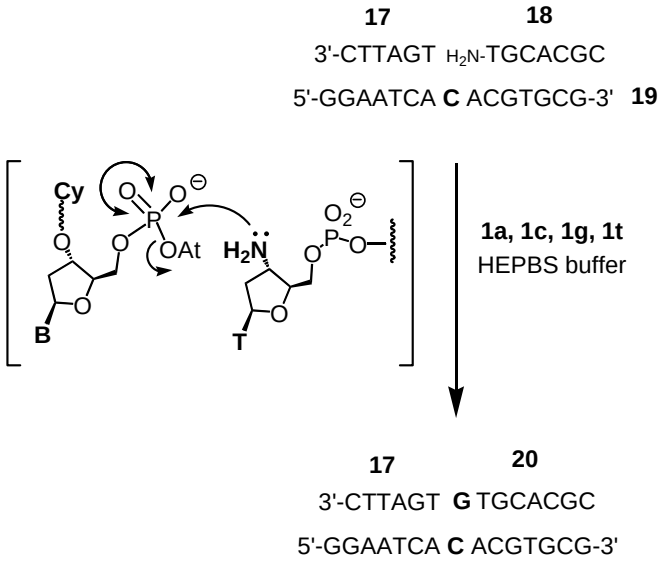


Figure S6. Kinetics of primer extension reactions with unlabeled OAt-esters.[3] Shown are the kinetics of the extension by the matched nucleotide. "Template A, C" etc. refers to templates **9a**, **9c** etc.

4. Primer Extension with Alternative Sequence Motif

The scheme below shows the components of the primer extension assay performed with the second, alternative sequence motif and the fluorophore-labeled OAt-esters of the four deoxynucleotides (**1a-1t**).



The brackets to the left of the arrow show the attack of amino-terminal primer **18** on the labeled and activated monomer. The reaction is templated by **19** and aided by oligonucleotide **17** as 'helper'. The assay is analogous to that shown in Scheme 2. Primer **18** was prepared analogously to oligonucleotide **8**, and unmodified **17** and **19** were prepared by conventional DNA synthesis. The assay was performed as described in General Protocol 4, using a total volume of 2.5 µL aqueous buffer solution , except that 0.2 M HEPBS (N-(2-hydroxyethyl)-piperazine-N'-(4-butanesulfonic acid) was used instead of HEPES. Again, the strand concentration was 36 µM and an equimolar mixture of the activated, fluorophore-labeled nucleotides **1a-t** was used (3.6 mM each).

The identity of the elongated primer was determined using mass spectrometry, as described in General Protocol 5. Figure S7, below, shows MALDI-TOF mass spectra prior to and after primer extension. It can be discerned that the correct nucleotide was added to **18** with high fidelity.

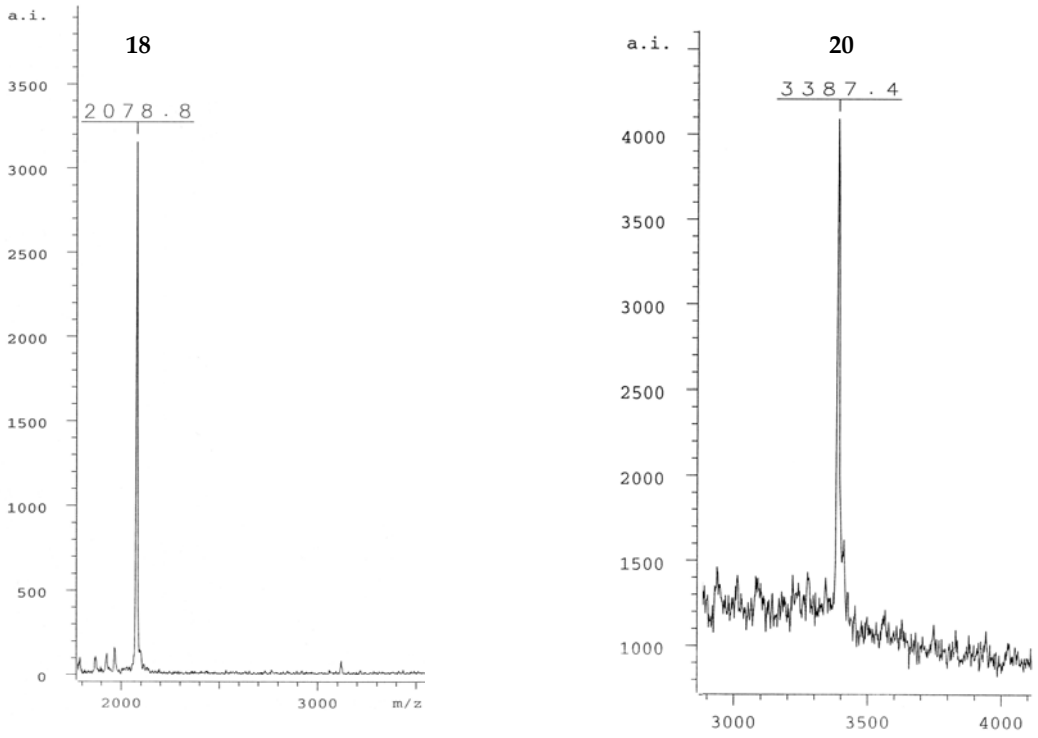


Figure S7. MALDI-TOF mass spectra primer **18** (left) and the extended primer **20** generated in the reaction with **1a-t** templated by **19**. Reaction conditions: 36 µM template (**19**), primer (**18**) and helper (**17**), equimolar mixture of fluorophore-labled OAt-esters **1a-t** (3.6 mM) in HEPBS buffer (HEPBS 0.2 M, $MgCl_2$ 0.08 M, NaCl 0.4 M, pH 8.9). The spectrum shown on the right was acquired from the reaction solution after 18.5 h reaction time at room temperature.

5. Detection of Labeled Oligonucleotides on Microarrays

Preparation of Microarrays (General Protocol 6).

For the preparation of microarrays, a protocol similar to that described in Dombi et al.[7] was used. The protocol is also similar to what was described by Dogan et al.[6] Prior to use, aldehyde slides were washed with water for 10 min, and then dried in a stream of argon. Solutions containing the lysine-terminated capture oligonucleotides were prepared as described earlier.[7] On each spot, a mixture of solutions 10 µM DNA, 30 µM 3,6,9-trioxydecylamine[7], 0.1 M MES buffer, 0.1 M glycerol (1:1:1:1, v/v) was spotted on the desired areas of the slide. The immobilization was performed for 60 min at r.t. in a humid chamber. Then, 0.5 µL of a solution of $NaBH_3CN$ (50 mM in PBS buffer) was added to the droplet, followed by reduction for 30 min. The slide was washed by first rinsing with 0.1x SSC/0.2 % SDS (25 mL) and then dipping in 0.1x SSC/0.2 % SDS (50 mL) and water (50 mL), and dried in a stream of argon. Remaining aldehyde groups on the surface were then reacted using a solution of 3,6,9-trioxydecylamine (50 µM) and $NaBH_3CN$ (50 mM) in PBS buffer for 2 h at r.t. Again, the slide was washed, first by rinsing with 0.1x SSC/0.2 % SDS (25 mL) and then by dipping into 0.1x SSC/0.2 % SDS (50 mL) and water (50 mL), followed by drying in a stream of argon.

Hybridization (General Protocol 7)

The solutions containing the Cy-labeled oligonucleotides (6 µL), 1 µM in DNA, in 2x SSC/0.2% SDS buffer were applied on the slide surface under cover slips. The slide was incubated for 1 h at r.t. in a humid chamber. Then, the slide was washed, first by rinsing with 1x SSC/0.2 % SDS (25 mL) and then by dipping into 1x SSC/0.2 % SDS (50 mL) for 1 min, followed by drying in a stream of argon.

Optical Read-Out

a) Calibration experiments involving Cy-labeled reference oligonucleotides

The cyanine dyes give different fluorescence intensities in scans of chip surfaces. Further, there is "cross talk" between the different channels due to partial overlap in the fluorescence spectra of the dyes. For a recent study that highlights these issues for a different sequencing application, the reader is referred to Metzker et al.[8] In the present case, the response for the different fluorophores was determined in independent calibration experiments employing Cy-labeled oligonucleotides synthesized separately. These were of the sequence 5'-Cyn-ATCGCAGTCAACCA-3', where n is 3, 3.5, 5, or 5.5, and were hybridized (1 µM DNA) to a microarray displaying capture oligonucleotide 5'-TGGTTGACTGCGAT-DP-Lys-3'[6,7] as described in General Protocol 7.

As mentioned under General, microarrays were scanned with an ArrayWoRx®e biochip reader (Applied Precision, Issaquah, WA, USA, obtained through GeneScan, Freiburg, Germany) with a resolution of '20000' and 'high SNR sensitivity'. The biochip reader has a polychromatic light source, a four color filter wheel, and a CCD camera detection system. The following filters were used (excitation wavelength ± bandwidth of transmission/emission wavelength ± bandwidth of transmission): Cy 3: (546 ±10 / 575 ± 30); Cy 3.5: (577 ± 10) / 615 ± 30); Cy 5: (640 ± 20) / 680 ± 30); Cy 5.5: (675 ± 20) / 725 ± 50). The filters were from Chroma Technology Corporation (Rockingham, USA). The intensity of the fluorescence signals was determined with the Array WoRx®e analysis software supplied with the chip reader. Images of the slides were created with NIH Image/Scion Image.[9] To compensate for the lower signal intensity for Cy5 and Cy5.5, the exposure time was increased five-fold for their channels. Under the optimized conditions, the relative signal intensities for individual dyes were within a factor of 2 of the average signal intensities. Appropriate correction factors for relative signal intensity and cross talk contribution were applied when quantifying.

Table S2: Cross talk between channels for detection of the four cyanine dyes under optimized conditions.

Channel	Exposure time[a] [sec.]	Signal respective dye (%) Cy3	Cy3.5	Cy5	Cy5.5
Cy3	*0.1*	100	12	2	2
Cy3.5	*0.1*	12	100	6	4
Cy5	*0.5*	<1	2	100	32
Cy5.5	*0.5*	<1	<1	32	100

[a] Exposure time, as set for the microarray scanner.

b) Analysis of products of chemical primer extension reactions with monomers 1a-t

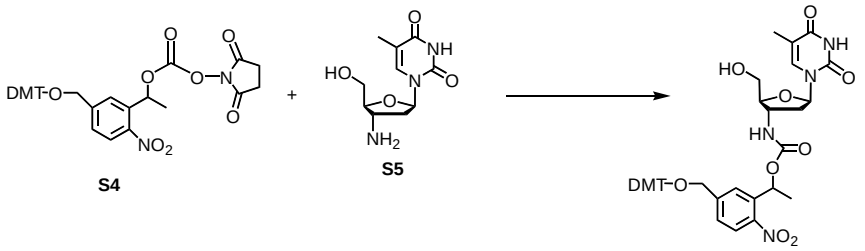
The microarrays were prepared as described in General Protocol 6, using capture 5'-Lys-DP-TAAAAGATACCATCAA-3' (**12**) (1 µL per spot, 10 µM DNA). Two spots per assay solution to be analyzed were generated. Samples were drawn and pretreated as described for MALDI-TOF analysis. The hybridization was performed as described in General Protocol 7, using solutions (6 µL) approx. 1 µM in DNA in 2x SSC/0.2% SDS buffer. Optical read-out via fluorescence scans was performed as described above, using the optimized conditions.

5. Assays with Monomers Featuring a Photolabile Linker

Synthesis of 13

Compound **S4** was prepared in 8 steps via a procedure similar to that of Seo, et al.[10] Details of the synthesis will be published elsewere.

5'-HO-dT-PC (**S3**)



3'-Aminothymidine (**S5**) was prepared through catalytic hydrogenation of AZT according to Lin et al.[11] 3'-Aminothymidine (**S5**) (220 mg, 0.9 mmol) was dissolved in dry DMF (5 mL), containing TEA (0.2 mL, 1.41 mmol) and then treated with carbonate **S4** (600 mg, 0.94 mmol). After stirring the solution for 12 h in the dark, CH_2Cl_2 (20 mL) was added, and the organic solution was washed with water (25 mL) and then with saturated bicarbonate solution (2x 15 mL). The organic layer was dried over Na_2SO_4, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica (CH_2Cl_2/MeOH 97:3) to yield the title compound as a yellowish solid. Yield 655 mg, 0.85 mmol (94 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ 9.4 (m, 1H), 7.93 (m, 1H), 7.65 (m, 1H), 7.60-7.21 (m, 11H), 6.87 (m, 4H), 6.30 (m, 1H), 6.18 (m, 1H), 5.86 (m, 1H), 4.36-4.21 (m, 3H), 3.92 (m, 1H), 3.80 (m, 6H), 3.62 (m, 1H), 3.10 (m, 1H), 2.33-2.22 (m, 2H), 1.86 (m, 3H), 1.63 (m, 3H).

^{13}C-NMR (125 MHz, $CDCl_3$): δ 163.9, 163.8, 158.6, 155.6, 155.5, 150.6, 155.5, 146.2, 146.0, 145.9, 144.6, 138.0, 137.8, 136.0, 135.9, 135.8, 135.7, 130.0, 128.0, 127.0, 126.4, 124.8, 124.7, 124.6, 113.3, 110.9, 86.9, 85.8, 85.6, 84.6, 84.5, 69.5, 64.7, 61.6, 61.5, 55.3, 49.9, 37.8, 37.5, 22.0, 12.5.

FAB-MS (3-NBA): m/z = 767.3.

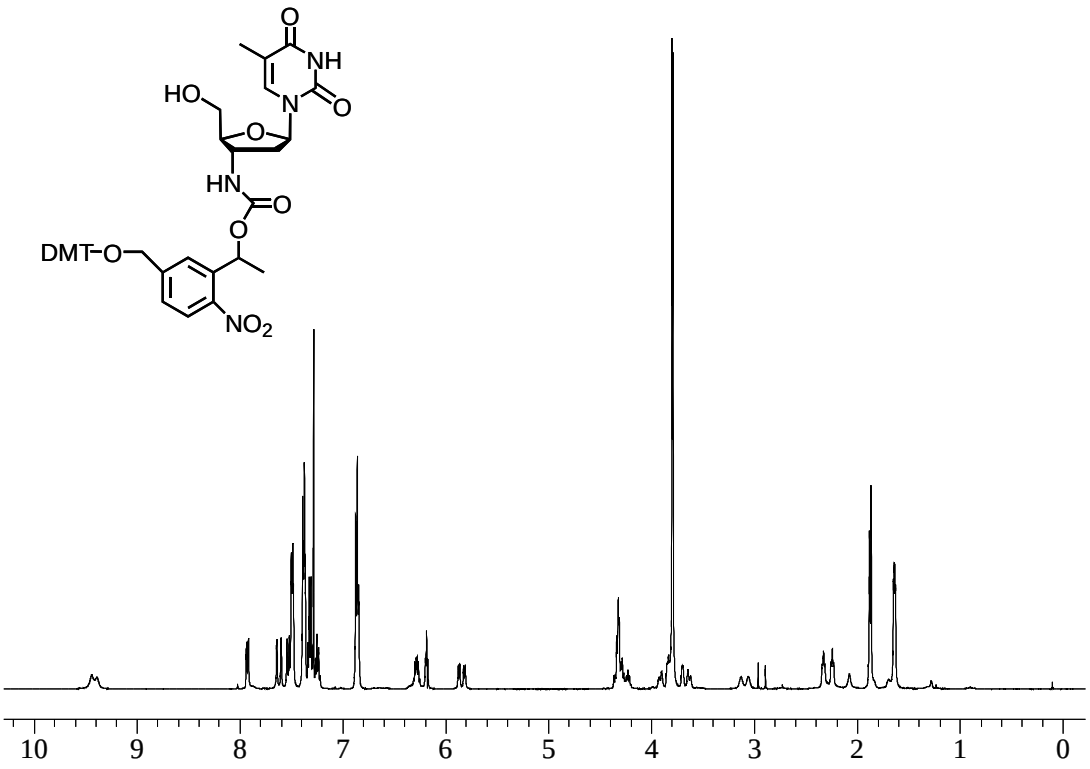
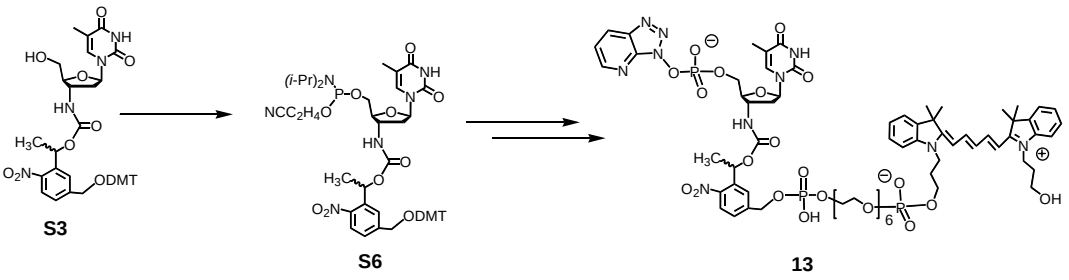


Figure S8. ^{1}H-NMR (500 MHz, $CDCl_3$) of compound **S3**.

OAt-P-dT-PC-Cy5 (13)

Compound **13** was prepared from **S3** through phosphitylation, followed by precipitation and solid phase phosphoramidite synthesis with the crude, following the synthetic scheme shown below.



Compound S6

All steps described below must be performed excluding UV light. A solution of anhydrous **S3** (77 mg, 0.1 mmol) and DIEA (46 µL, 0.28 mmol) in CH_3CN (2 mL) was treated with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (32 µL, 0.14 mmol) at 0 °C in the dark with stirring. The cooling bath was removed, and after 1 h additional 2-cyanoethyl-*N*,*N*-

diisopropylchlorophosphoramidite (32 µL, 0.14 mmol) was added. After 30 min, the mixture was reduced to dryness *in vacuo*, followed by addition of dichloromethane (20 mL). The resulting solution was washed with satd. $NaHCO_3$ solution (2 x 20 mL), dried over Na_2SO_4, reduced to dryness, coevaporated with CH_3CN (2 x 5 mL) and reduced to dryness, followed by drying at 0.1 Torr. The residue was taken up in dichloromethane (1 mL) and dripped into a solution of triethylamine (0.25 mL) in n-pentane (30 mL), resulting in an amorphous precipitate. The supernatant was aspired and the residue was dried at 0.1 Torr. Compound **S6** was thus obtained as a slightly yellow foam (83 mg, 0.085 mmol, 85 %).

Compound 13

Crude **S6**, as obtained by precipitation, was employed for solid phase synthesis of the nucleotide precursor of **13**, using a procedure analogous to that shown in Scheme 1 and the conditions given in General Protocols 1 and 2. The product eluted between 30 and 45 % of acetonitrile from the Sep-Pak cartridge, yield 5 %. MALDI-TOF MS for nucleotide precursor of **13** (linear, positiv, TC21): calcd for $C_{63}H_{88}N_6O_{25}P_3$ ($[M+H]^+$): 1422.3, found 1422.1.

The 5'-phosphate thus obtained was then converted to OAt-ester **13** using the conditions of General Protocol 3.

MALDI-TOF MS (linear, positive): calcd for $C_{68}H_{90}N_{10}O_{25}P_3$ ($[M+H]^+$): 1540.4, found 1540.9.

Chemical Primer Extension Involving OAt-P-dT-PC-Cy5 (13)

Non-enzymatic, template-directed primer extension reaction was carried out in 2.5 µL of an aqueous buffer solution at r.t. following General Protocol 6, except for using OAt-P-dT-PC-Cy5 (**13**) instead of OAt-P-dT-Cy5 (**1t**) and unlabeled OAt-esters of dAMP, dCMP, and dGMP (**16a-g**) instead of **1a-g** as competitor monomers. Sampling and MALDI analysis followed the protocols given under General Protocol 5. It should be noted that the N_2 laser of the MALDI-TOF mass spectrometer leads to partial cleavage of the photolabile linker during mass spectrometry, as seen in the small signal for **15** in the mass spectrum of the assay solution prior to UV irradiation.

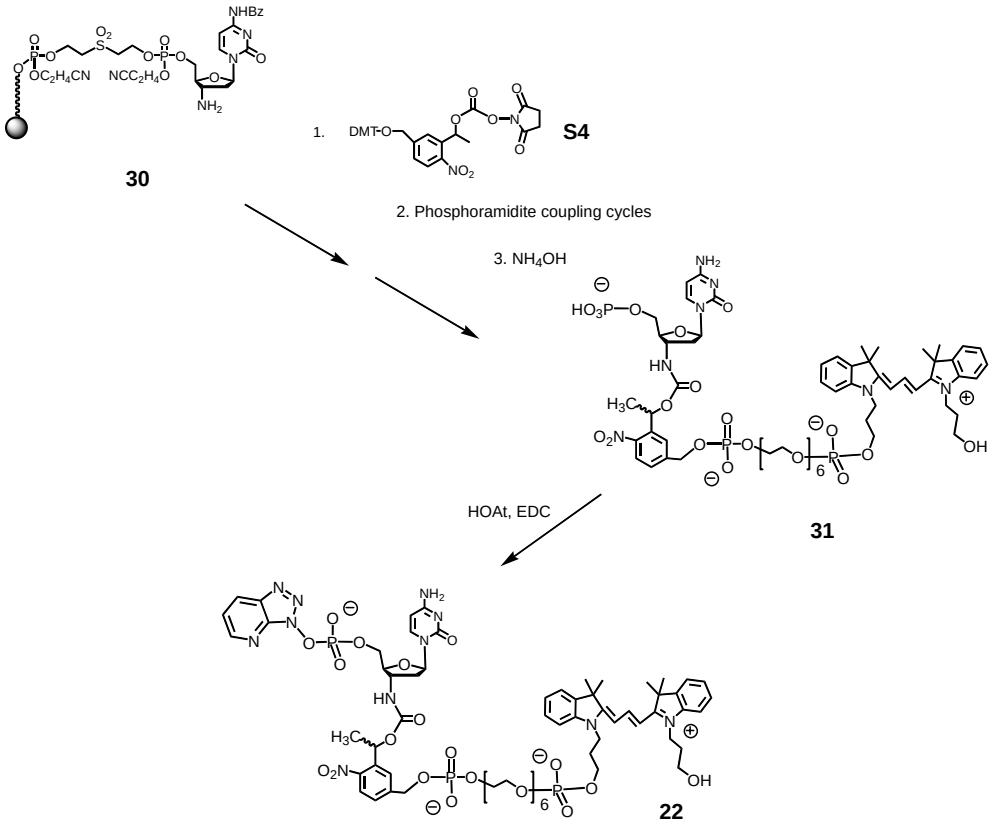
Photochemical cleavage of the linker in 14

A sample of 1 µL of the solution obtained from the primer extension reaction with subsequent micro column treatment to remove excess **13** in a polypropylene vessel was irradiated with UV-light (254 nm) from a hand-held lamp for illumination TLC plates for 5 min. The solution was then analyzed via MALDI TOF mass spectrometry as detailed in General protocol 5.

Dual Primer Extension Involving OAt-P-dC-PC-Cy3 (22) and OAt-P-dC (16c)

Synthesis of OAt-P-dC-PC-Cy3

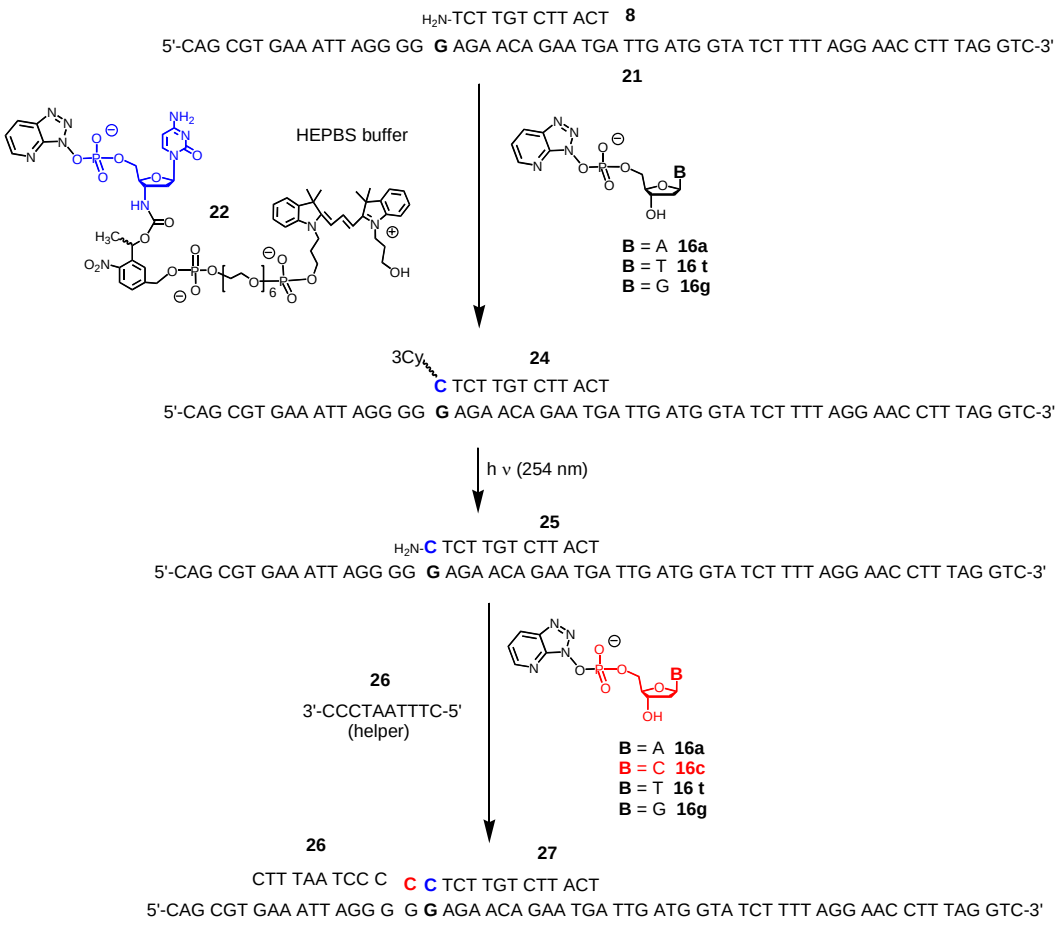
Compound **22** is a deoxycytidine analog of **13**. It was prepared via solid phase synthesis of **31** on controlled pore glass, followed by activation in solution, as shown below.



The details of the synthesis of **22** will be published elsewhere. Briefly, a solid phase synthesis on 2 µmol scale similar to that specified in General Protocol 1 was performed to generate **31**. This synthesis involved the 5'-phosphoramidite building block of 3'-amino-2',3'-dideoxycytidine (Transgenomic, Paisley, Scotland) and carbonate **S4**, as well as the reagents used for preparing **1a-1t**. The aqueous deprotection liberating **31** was performed under the conditions given in General Protocol 2. The product (**31**) eluted from the Sep-Pak cartridge between 30 and 40 % acetonitrile; yield 25 %. MALDI-TOF MS of **31** (linear positive): calcd for $C_{60}H_{81}N_7O_{24}P_3$: 1376.3, found 1377.3. Nucleotide **31** thus obtained was converted to OAt-ester **22** using the conditions of General Protocol 3. MALDI-TOF MS for **22** (linear positive): calcd for $C_{65}H_{84}N_{11}O_{24}P_3$: 1495.4, found 1494.2.

Dual Primer Extension Assays

The exploratory assay performed to demonstrate the feasibility of multiple extension reactions is shown schematically below. The first nucleotide added to primer **8** is given in blue, the second nucleotide appended is given in red.



The assay steps analogous to those detailed for the extension of the same primer with **13** (Scheme 3). The reaction was carried out in 2.5 µL of an aqueous buffer similarly to General Protocol 4. Compound **22 (**18mM**)** and an equimolar mixture of competitors **16a,t,g** (3.6 mM each) were reacted in the presence of template **21** to give extended primer **24**. Briefly, to a stock solution of the buffer (1 µL; HEPBS 0.5 M, $MgCl_2$ 0.2 M, NaCl 1 M, pH 8.9) an aliquot (0.25 µL) of each of the stock solutions of template (**21**) and primer (**8**) was added. To this solution was added water (0.25 µL), and 0.5 µL of a mixture of OAt-P-dC-PC-Cy3 (**22**) and the OAt-activated monomers (**16a,t,g**). The concentration of the oligonucleotides was 36 µM. The final salt concentrations in the buffer were HEPBS 0.2 M, $MgCl_2$ 0.08 M, NaCl 0.4 M. After extension for 22 h at room temperature and micro column treatment, the solution was irradiated with UV-light (254 nm) from a hand-held lamp for 5 min, resulting in the formation of **25**, as confirmed by MALDI TOF mass spectrometry (*vide infra*). The mixture was

lyophilized from water, and fresh assay buffer (HEPBS), helper **26**, and activated monomers (**16a-t**) were added. There second primer extension reaction was allowed to proceed over night under the same conditions as the first step (room temperature). Samples were drawn to document each stage of the assay. MALDI analysis (Figure S9) followed General Protocol 5.

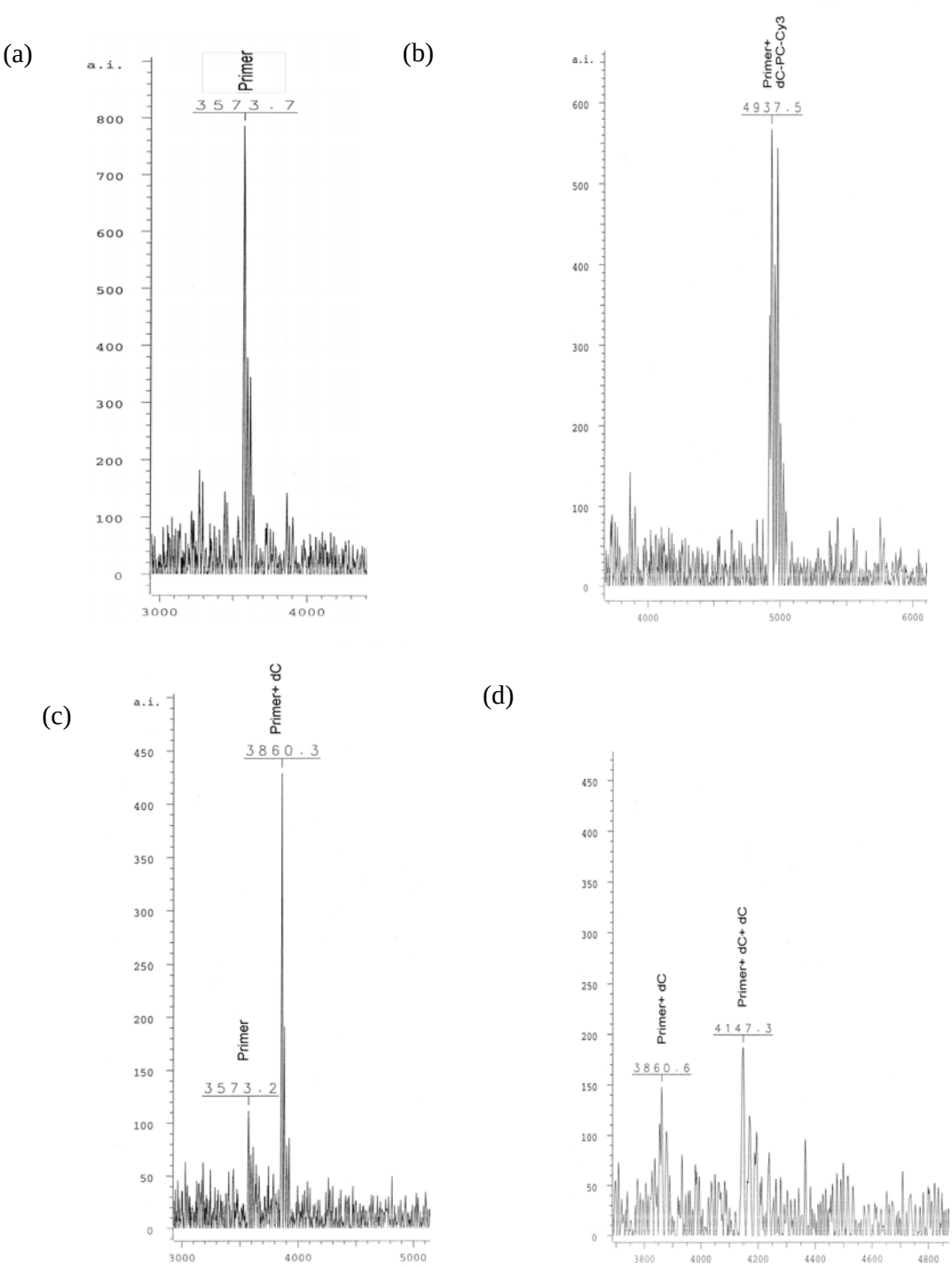


Figure S9. MALDI-TOF mass spectra of dual extension assay from samples drawn prior to (a), and after extension of **8** with **22** (b), followed by photolysis (c), and extension with an additional dC residue (d). Repeated sampling depletes the solution, leading to a loss of signal intensity as the assay progresses.

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