# Supporting Information

## Azide and *trans*-Cyclooctene dUTPs: Incorporation into DNA Probes and Fluorescent Click-Labelling

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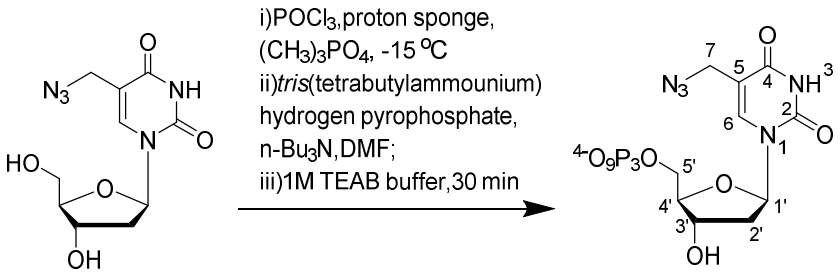
## S1: Deoxynucleotide triphosphate synthesis: General information

All reagents were purchased from Sigma-Aldrich, Alfa Aesar, Acros Organics and Fisher Scientific and used without purification with the exception of dichloromethane and N, N-diisopropylethylamine which were dried over activated molecule sieves overnight. Chemical transformations were carried out under an atmosphere of argon using oven-dried glassware. Thin layer chromatography (TLC) was performed using Merck Kieselgel 60 F24 silica gel plates (0.22 mm thickness, aluminium backed) and the compounds were visualised by irradiation at 254 nm or by staining with anisaldehyde. Column chromatography was carried out under pressure using Merck Kieselgel Si 60 (40-63 micron) silica.

$^{1}$H NMR spectra were measured at a 400 MHz on a Bruker AVIII400 spectrometer, or at 500 MHz on a Bruker AVII500 spectrometer. The $^{13}$C NMR spectra were measured at 126 MHz on a Bruker AVII500 spectrometer with a Cryoprobe. The $^{31}$P NMR spectra were recorded on a Bruker AVIII400 or AVII500 spectrometer with a Cryoprobe at 162 MHz and 202 MHz. Chemical shifts are given in ppm, and *J* values are quoted in Hz. All spectra were internally referenced to the appropriate residual undeuterated solvent signal, assignment of the compounds was aided by COSY ($^{1}$H-$^{1}$H), HSQC-DEPT and HSBC ($^{1}$H-$^{13}$C) experiments.

All low-resolution mass spectra were recorded using electrospray ionisation on a Micromass platform 1 spectrometer in HPLC grade acetonitrile. Electrospray mass spectra of deoxynucleotide triphosphates were recorded in water on a Bruker microTOF focus ESI-TOF instrument in ES$^{-}$ mode.

## 5-Azidomethyl-2´-deoxyuridine-5´-triphosphate (AM dUTP)




**5-azidomethyl dU** **AM dUTP**

5-Azidomethyl deoxyuridine[1] (100.0 mg, 0.353 mmol) was dried over KOH together with proton sponge (113.3 mg, 0.528 mmol, 1.5 eq), activated molecular sieves and *tris*(tetrabutylammonium) hydrogen pyrophosphate. Trimethylphosphate (2.5 mL) was dried over molecular sieves overnight and then added with proton sponge to 5-azidomethyl-deoxyuridine under argon at -15 °C. Phosphorus oxychloride (40 µL, 0.426 mmol, 1.2 eq) was added dropwise. After 30 min, a mixture of *tris* (tetrabutylammonium) hydrogen pyrophosphate (1.5 g, 1.66 mmol, 5 eq) in DMF (1.6 mL) and tributylamine (0.339 mL) was added and the reaction was stirred at -15 °C for 3 h. 1.0 M Triethylammonium bicarbonate (TEAB) buffer (pH 7.5, 7.5 mL) was added to quench the reaction and the solvent was removed *in vacuo*. The crude reaction mixture was diluted in 3 ml water and mixed with 300 µL of 3 M NaCl solution in water and 9 ml ethanol to precipitate the triphosphate. To give more product, the precipitation was repeated three times on the supernatant (the solvent of the supernatant was removed and redissolved in water every time) until further precipitation did not yield any product. The precipitated pellet was dissolved in water and purified by RP-HPLC using a Phenomenex HPLC column (Gemini-NX 10µ C18 110A AXIA, 250 x 21.2 mm). Buffer A: 0.1 M TEAB (pH 7.5), B: 40 % acetonitrile in 0.1 M TEAB (pH 7.5). Gradient: 3.5 % - 30 % buffer B in 50 min. The reaction was monitored by TLC (2-propanol:ammonia:water, 2:1:1) and stained with anisaldehyde. Pure 5-azidomethyl-2´-deoxyuridine triphosphate (26.5 µmol, 7.5 %) was obtained. The concentration c was calculated by $A = \varepsilon Lc$ ($\lambda_m$ = 265 nm, $\varepsilon$ = 9720, L = 1 cm, water, 25 ºC). The product was characterized by mass spectrometry.

MS: $M_w$ = 522.991 ($C_{10}H_{16}N_5O_{14}P_3$), $[M-H]^-$ = 521.983, Found $[M-H]^-$= 521.995

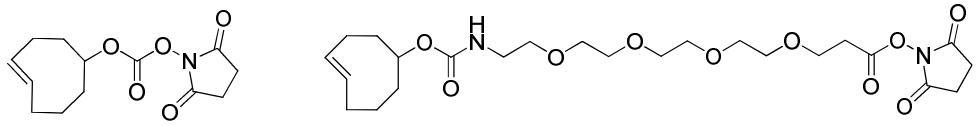
$^1$H NMR (400MHz , $D_2O$) δ = 8.08 (s, 1 H, H-6), 6.33 (t, *J* = 6.4 Hz, 1 H, H-1´), 4.72 - 4.68 (m, 1 H, H-3´), 4.33 - 4.16 (m, 5 H, H-4´, H-5´, H-7), 3.20 (q, *J* = 7.3 Hz, $Et_2NH^+C\boldsymbol{H}_2CH_3$), 2.44 - 2.38 (m, 2 H, H-2´), 1.28 (t, *J* = 7.3 Hz, $Et_2NH^+CH_2C\boldsymbol{H}_3$) ppm

$^{13}$C NMR (126MHz, $D_2O$) δ = 141.0 (C6), 109.6 (C5), 85.7 (C4´), 85.2 (C1´), 70.0 (C3´), 65.0 (C5´), 47.1 (C7), 46.6 ($Et_2NH^+\boldsymbol{C}H_2CH_3$), 38.7 (C2´), 8.2 ($Et_2NH^+CH_2\boldsymbol{C}H_3$) ppm

$^{31}$P NMR (162 MHz, $D_2O$) δ = -6.5 (d, *J* = 21.5 Hz, $P_\gamma$), -11.5 (d, *J* = 19.6 Hz, $P_\alpha$), -22.6 (app t, *J* = 21.5 Hz, $P_\beta$) ppm

## 5-*Trans*-cyclooctene-2´-deoxyuridine-5´-triphosphates

The freeze-dried 5-aminopropargyl-2´-deoxyuridine-5´-triphosphate[2] (2.3 µmol) was dissolved in 120 µL 1.0 M TEAB buffer (pH 7.5). TCO-S NHS ester (1.5 mg) or TCO-L NHS ester (2 mg) in 100 µL DMF was added to the solution. The mixture was kept at 55 ºC for 4 h and the solvent was removed *in vacuo*. The products were purified by RP-HPLC using a Phenomenex HPLC column (Luna 10µ C8 100Å, 10x250 mm). Eluent A: 0.1 M TEAB buffer (pH 7.5), B: 60 % acetonitrile in 0.1 M TEAB (pH 7.5). Gradient: 3.5 % - 60 % buffer B in 20 min. The pure products were characterized by mass spectrometry and NMR (below). TCO-S dUTP was isolated in 53 % yield (1.2 µmol) and TCO-L dUTP was isolated in 64 % yield (1.5 µmol). The two active esters were purchased from Jena Bioscience.



TCO-S NHS TCO-L NHS

**Figure S-1.** Structures of *trans*-cyclooctene NHS esters

**TCO-S dUTP**


**5-aminopropargyl dUTP** **TCO-S dUTP**

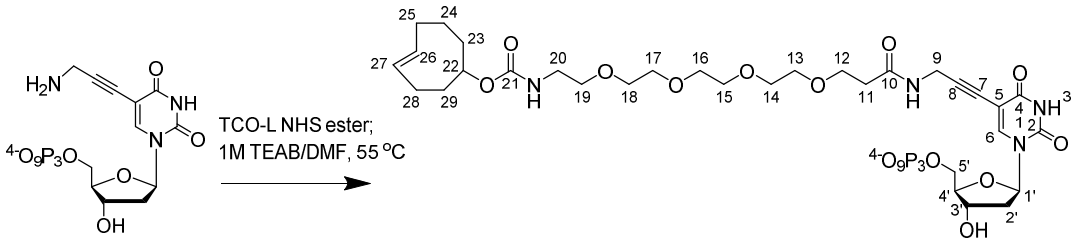
MS: $M_w$= 673.084 ($C_{21}H_{30}N_3O_{16}P_3$), $[M-H]^-$ = 672.077, Found $[M-H]^-$= 672.080

$^1$H NMR (500MHz, $D_2O$) δ = 8.07 (s, 1 H, H-6), 6.23 (t, *J* = 6.5 Hz, 1 H, H-1´), 5.66 (ddd, *J* = 4.7, 9.1, 16.2 Hz, 1 H, H-16), 5.52 (ddd, *J* = 2.8, 10.9, 16.2 Hz, 1 H, H-15), 4.61 (app dd, *J* = 4.1, 9.3 Hz, 1 H, H-3´), 4.33 - 4.25 (m, 1 H, H-11), 4.24 - 4.12 (m, 3 H, H-4´, H-5´), 4.05 (s, 2 H, H-9), 3.15 (q, *J* = 7.3 Hz, $Et_2NH^+C\boldsymbol{H}_2CH_3$), 2.41 - 2.23 (m, 5 H, H-2´, H-17, H-12a ), 2.04 - 1.82 (m, 4 H, H-14, H-12b, H-13a), 1.73 - 1.51 (m, 3 H, H-18, H-13b), 1.23 (t, *J* = 7.3 Hz, $Et_2NH^+CH_2C\boldsymbol{H}_3$) ppm

$^{13}$C NMR (126MHz, $D_2O$) δ = 144.6 (C6), 135.7 (C16), 133.4 (C15), 99.1 (C5), 85.5 (C4′, C1′), 82.4(C11), 70.0 (C3′), 65.0 (C5′), 46.6 ($Et_3NH^+\boldsymbol{C}H_2CH_3$), 40.3 (C14), 38.6 (C2′), 37.8 (C18), 33.7 (C12), 32.0 (C17), 30.5 (C13, C9), 8.2 ($Et_2NH^+CH_2\boldsymbol{C}H_3$) ppm

$^{31}$P NMR (202MHz, $D_2O$) δ = -5.1 - -5.4 (m, $P_\gamma$), -9.9 - -10.1 (m, $P_\alpha$), -20.9 - -21.3 (m, $P_\beta$) ppm

**TCO-L dUTP**




**5-aminopropargyl dUTP** **TCO-L dUTP**

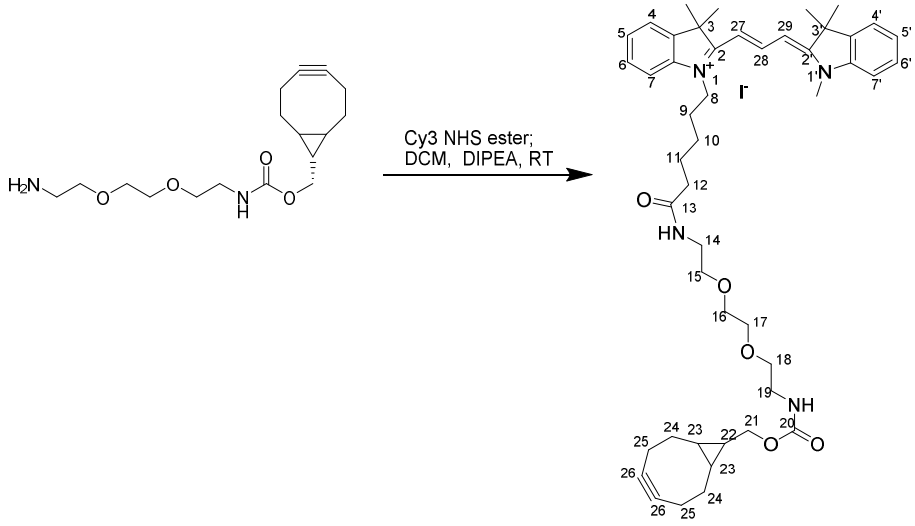
MS: $M_w$= 920.226 ($C_{32}H_{51}N_4O_{21}P_3$), $[M-H]^-$ = 919.218, Found $[M-H]^-$= 919.231

$^1$H NMR (500MHz , $D_2O$) δ = 8.13 (s, 1 H, H-6), 6.22 (t, *J* = 6.5 Hz, 1 H, H-1′), 5.69 - 5.57 (m, 1 H, H-27), 5.56 - 5.45 (m, 1 H, H-26), 4.65 - 4.58 (m, 1 H, H-3′), 4.27 - 4.12 (m, 6 H, H-22, H-5′, H-4′, H-9), 3.77 (t, *J* = 6.0 Hz, 2 H, H-12), 3.59-6.66 (m, 12 H, H-13 to H-18), 3.54 (t, *J* = 5.2 Hz, 2 H, H-19), 3.24 (t, *J* = 5.2 Hz, 2 H, H-20), 3.16 (q, *J* = 7.3 Hz, $Et_2NH^+C\boldsymbol{H}_2CH_3$), 2.54 (t, *J* = 6.0 Hz, 2 H, H-11), 2.40 - 2.32 (m, 2 H, H-2′), 2.32 - 2.22 (m, 3 H, H-28, H-23a), 2.00 - 1.83 (m, 4 H, H-25, H-23b, H-24a), 1.67 - 1.51 (m, 3 H, H-24b, H-29), 1.23 (t, *J* = 7.3 Hz, $Et_2NH^+CH_2C\boldsymbol{H}_3$) ppm

$^{13}$C NMR (126MHz , $D_2O$) δ = 173.8 (C10), 158.3 (C4), 144.9 (C6), 135.6 (C27), 133.3 (C26), 99.1 (C5), 89.7 (C7, C8), 85.6 (C4′, C1′), 81.8 (C22), 70.1 (C3′), 69.6, 69.5, 69.5 and 69.4 (C13, C14, C15, C16, C17, C18, C19), 66.6 (C12), 65.0 (C5′), 46.6 ($Et_3NH^+\boldsymbol{C}H_2CH_3$), 40.4 (C25), 39.9 (C20), 38.7 (C2′), 37.8 (C29), 35.9 (C11), 33.7 (C23), 32.0 (C28), 30.6 (C24), 29.6 (C9), 8.2 ($Et_2NH^+CH_2\boldsymbol{C}H_3$) ppm

$^{31}$P NMR (162MHz, $D_2O$) δ = -8.0 (br.s, $P_\gamma$), -11.4 (d, *J* = 19.6 Hz, $P_\alpha$), -22.6 (br.s, $P_\beta$) ppm

## Cy3-BCN synthesis




**Cy3 BCN**

Cy3 NHS ester (30 mg, 0.051 mmol, 1 eq) was dissolved in anhydrous DCM (2 mL), then anhydrous DIPEA (0.030 mL, 0.172 mmol, 3.4 eq) and the above BCN amine (purchased from SynAffix, 25 mg, 0.077 mmol, 1.5 eq) were added. The reaction mixture was stirred overnight at RT, diluted with DCM then washed with water and potassium iodide. The organic layer was dried over sodium sulfate, filtered and evaporated. The product was dissolved in 2 mL DCM and added to cold diethyl ether to afford a red precipitate. The precipitate was purified by column chromatography (0-5 % MeOH/DCM) and the product (23 mg, 0.026 mmol) was obtained in 51 % yield.

LRMS [ESI+, $CH_3CN$] $M_w$ = 890.9 ($C_{47}H_{63}IN_4O_5$), $[M\text{-}I^-]^+$ = 763.5; found: 763.1

$^1$H NMR (500MHz ,$CDCl_3$) δ = 8.44 (app t, *J* = 13.4 Hz, 1 H, H-28), 7.44 - 7.38 (m, 3 H, ArH, H-27), 7.36 (d, *J* = 7.3 Hz, 2 H, ArH ), 7.30 - 7.22 (m, 3 H, ArH, H-29), 7.13 (dd, *J* = 2.5, 7.9 Hz, 2 H, ArH), 6.88 (br.s, N-H), 5.36 (br.s, N-H), 4.18 (t, *J* = 7.9 Hz, 2 H, H-8), 4.14 (d, *J* = 8.2 Hz, 2 H, H-21), 3.83 (s, 3 H, N-$CH_3$), 3.65 - 6.62 (m, 4 H, H-16, H-17), 3.60 (t, *J* = 5.6 Hz, 2 H, H-15), 3.58 (t, *J* = 5.0 Hz, 2 H, H-18), 3.46 (app q, *J* = 5.6 Hz, 2 H, H-14), 3.39 (app q, *J* = 5.0 Hz, 2 H, H-19), 2.37 (t, *J* = 7.1 Hz, 2 H, H-12), 2.32 - 2.16 (m, 6 H, H-25, H-24a), 1.94 - 1.85 (m, 2 H, H-9), 1.84 - 1.75 (m, 2 H, H-11 ), 1.73 (s, 6 H, 2 x $CH_3$), 1.71 (s, 6 H, 2 x $CH_3$), 1.70 - 1.65 (m, 2 H, H-10), 1.63 - 1.52 (m, 2 H, H-24b), 1.40 - 1.31 (m, 1 H, H-22), 0.99 - 0.87 (m, 2 H, H-23) ppm

$^{13}$C NMR (126MHz , $CDCl_3$) δ = 174.6 and 174.1 (C2, C2'), 174.0 (C13), 157.3 (C20), 151.2 (C28), 143.2, 142.2, 141.0 and 140.9 (4 x quaternary C), 129.4 and 129.3 (C4, C4'), 125.8 and 125.7 (C6, C6'), 122.5 and 122.4 (C7, C7'), 111.4 and 111.2 (C5, C5'), 105.6 (C27), 104.9 (C29), 99.3 (C26), 70.8, 70.6, 70.5 and 70.2 (C15, C16, C17, C18), 63.1 (C21), 49.4

and 49.2 (C3, C3'), 45.3 (C8), 41.2 (C19), 39.4 (C14), 36.9 (C12), 33.4 (N-$CH_3$), 29.5(C24), 28.6(4 x $CH_3$), 28.5 (C9), 27.7 (C10), 25.9 (C11), 21.9 (C25), 20.5 (C23), 18.3 (C22) ppm

## S2: General method for oligonucleotide synthesis and purification

Standard DNA phosphoramidites, solid supports, and additional reagents were purchased from Link Technologies and Applied Biosystems Ltd. All oligonucleotides were synthesized on an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 1.0 μmol scale phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by the automated trityl cation conductivity monitoring facility and in all cases were >98.0 %. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling time for normal A, G, C, and T monomers was 40 s. Cleavage of oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 °C.

The building blocks for the RNA analogues were prepared using 2´-TBS protected RNA phosphoramidite monomers with *t*-butylphenoxyacetyl protection of the A, G and C nucleobases and unprotected U (Sigma-Aldrich). A solution of 0.3 M benzylthiotetrazole (BTT) in acetonitrile (Link Technologies) was used as the coupling agent, *t*-butylphenoxyacetic anhydride was employed as the capping agent and 0.1 M iodine as the oxidizing agent (Sigma-Aldrich). All RNA phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use, and the coupling time for all monomers was 10 min. Stepwise coupling efficiencies were determined by automated trityl cation conductivity monitoring and in all cases were >96 %. Cleavage of oligonucleotides from the solid support and deprotection were achieved by exposure to concentrated aqueous ammonia/ethanol (3/1 v/v) for 2 h at room temperature followed by heating in a sealed tube for 45 min at 55 °C. After cleavage from the solid support and deprotection of the nucleobases and phosphotriesters, RNA oligonucleotides were concentrated to a small volume *in vacuo*, transferred to 15 mL plastic tubes and freeze dried. The residues were dissolved in DMSO (300 μL) and triethylamine trihydrofluoride (300 μL) was added after which the reaction mixtures were kept at 65 °C for 2.5 h. Sodium acetate (3 M, 50 μL) and butanol (3 mL) were added with vortexing and the samples were kept at -80 °C for 30 min then centrifuged at 4 °C at 13,000 rpm for 10 min. The supernatant was decanted and the precipitate was washed twice with ethanol (0.75 mL) then dried under vacuum.

The fully deprotected oligonucleotides (DNA or RNA) were purified by reversed-phase HPLC on a Gilson system using a Luna 10μ C8 100Å pore Phenomenex 10x250 mm column

with a gradient of acetonitrile in ammonium acetate, in case of DNA, (0 % to 50 % buffer B over 20 min, flow rate 4 mL/min), (buffer A: 0.1 M ammonium acetate, pH 7.0, buffer B: 0.1 M ammonium acetate, pH 7.0, with 50 % acetonitrile). Elution was monitored by UV absorption at 295 nm. After HPLC purification, oligonucleotides were desalted using NAP-10 columns (GE Healthcare). For HPLC purification of RNA, triethylammonium bicarbonate (TEAB) buffer was used (buffer A: 0.1 M triethylammonium bicarbonate, pH 7.5, buffer B: 0.1 M triethylammonium bicarbonate, pH 7.5, with 50 % acetonitrile). The fractions from HPLC were evaporated without need for additional desalting.

All oligonucleotides were characterized by negative-mode electrospray HPLC-mass spectrometry, using a Bruker micro-TOF mass spectrometer and an Acquity UPLC system with a BEH C18 1.7 μm column (Waters). A gradient of acetonitrile in triethylamine (TEA) and hexafluoroisopropanol (HFIP) was employed, increasing from 0 % to 70 % buffer B over 8 min, with a flow rate of 0.2 mL/min (buffer A: 8.6 mM TEA, 200 mM HFIP in 5 % methanol/water; buffer B: 20 % buffer A in Methanol). Raw data were processed and deconvoluted using the Data Analysis function of the Bruker Daltronics Compass™ 1.3 software package.

## S3: Primer extension experiments

Primer extension reactions and reverse transcription reactions were conducted on BIO-RAD T100$^{TM}$ Thermal Cycler. Gel images were taken using Syngene G: BOX with the gene-snap imaging software.

### Primer extension using Klenow (template T1)

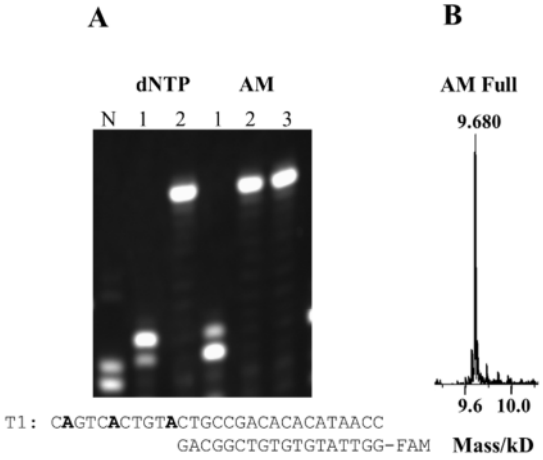



**Figure S-2.** Successful primer extension using AM dUTP with Klenow polymerase, primer P4 and template T1. 3.2 nmol of each triphosphate was used unless otherwise stated. A). Lane N, primer and template with dATP, dCTP and dGTP but without dTTP; dNTP lanes, unmodified triphosphate reactions; AM lanes, modified AM dUTP reactions; Lane 1, dTTP or modified dUTP (extra base incorporation was observed); Lane 2, dTTP or modified dUTP + dATP + dCTP + dGTP; Lane 3, dTTP + modified dUTP (1.6 nmol of each) + dATP + dCTP + dGTP (20 % PAGE gel). B. Mass spectra of fully extended primers: AM dUTP full length product (calc: 9679, found: 9680).

These PAGE and MS experiments indicate that Klenow large fragment polymerase efficiently incorporated AM dUTP into template T1.

Primer extension using GoTaq (template T1 and T2)

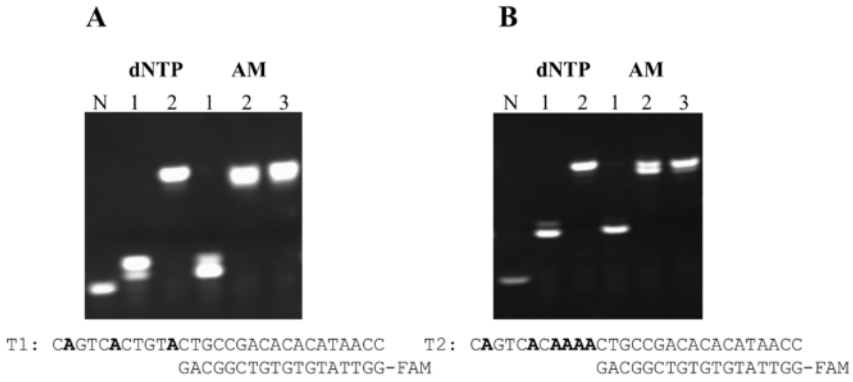



**Figure S-3**. Successful primer extension using AM dUTP with GoTaq DNA polymerase. 3.2 nmol of each triphosphate was used unless otherwise stated. A). Reaction with primer P4 and template T1; B). Reaction with primer P4 and template T2. Lane N, primer and template without dNTPs; dNTP lanes, unmodified triphosphate reactions; AM lanes, modified AM dUTP reactions; Lane 1, dTTP or modified dUTP (extra base incorporation was observed); Lane 2, dTTP or modified dUTP + dATP + dCTP + dGTP; Lane 3, dTTP + modified dUTP (1.6 nmol of each) + dATP + dCTP + dGTP (20% PAGE gel).

These PAGE experiments indicate that GoTaq polymerase efficiently incorporated AM dUTP into both template T1 and T2. GoTaq polymerase added an extra dA nucleotide to the fully extended products.

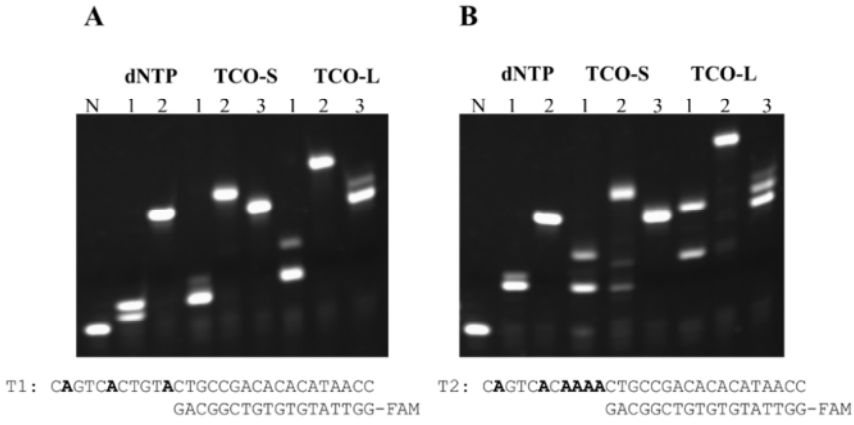



**Figure S-4.** Successful primer extension using TCO dUTPs with GoTaq DNA polymerase. 3.2 nmol of each triphosphate was used unless otherwise stated. A). Reaction with primer P4 and template T1; B). Reaction with primer P4 and template T2. Lane N, primer and template without dNTPs; dNTP lanes, unmodified triphosphate reactions; TCO lanes, modified TCO dUTP reactions; Lane 1, dTTP or modified dUTP (extra base incorporation was observed); Lane 2, dTTP or modified dUTP + dATP + dCTP + dGTP; Lane 3, dTTP + modified dUTP (1.6 nmol of each) + dATP + dCTP + dGTP (20% PAGE gel).

These PAGE experiments indicate that GoTaq polymerase successfully incorporated TCO dUTPs into both template T1 and T2. GoTaq polymerase added an extra dA nucleotide to the fully extended products.

## Primer extension using GoTaq (template T3)

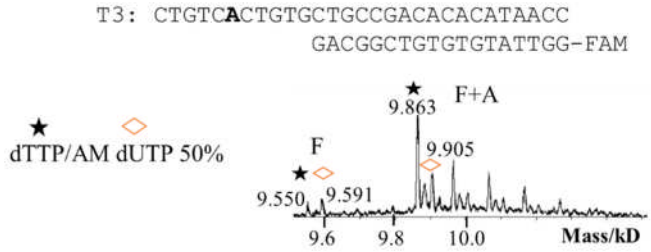



**Figure S-5.** Mass spectrum of primer extension reaction using GoTaq polymerase with primer P4 and template T3. 3.2 nmol of each triphosphate was used unless otherwise stated. AM dUTP (1.6 nmol) + dTTP (1.6 nmol) + dATP + dCTP + dGTP. dNTP full length product (F, calc: 9550, found 9550; F+A, calc: 9864, found: 9863); AM dUTP full length product (F, calc: 9591, found: 9591; F+A, calc: 9905; found: 9905 ). F = full length extension product; F+A = full length extension product plus dA.

These MS experiments indicate that AM dUTP competes with natural dTTP and is incorporated into template T3 which has only one incorporation site. GoTaq polymerase adds an extra dA nucleotide to the fully extended products. From the higher relative intensity of dTTP peaks compared to AM dUTP peaks, it is clear that GoTaq polymerase preferentially incorporates dTTP.

## Fluorescent labelling of primer extension products

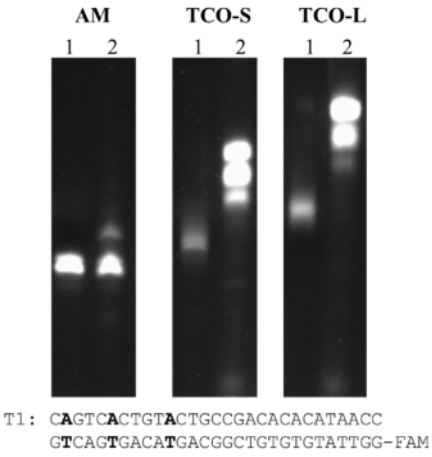



**Figure S-6.** Labelling reactions of primer extension products from P4 and T1 using GoTaq polymerase for 1 h (6.4 nmol). AM dUTP products were labelled with Cy3-BCN. TCO dUTP products were labelled with 6-methyl-tetrazine-sulfo-Cy3. AM lanes, modified AM dUTP reactions; TCO lanes, modified TCO dUTP reactions; Lane 1, not labelled; Lane 2, labelled products (20 % PAGE gel). After 4 h the AM dUTP product was 40 % labelled (not shown).

These PAGE experiments indicate that labelling TCO dUTP fully-extended products with 6-methyl-tetrazine-sulfo-Cy3 is more efficient than labelling AM dUTP fully-extended products with Cy3-BCN. Two or three sites are labelled for TCO dUTPs products after 1 h reaction, whereas for AM dUTP, intense non-labelled bands remained.

# S4: Reverse transcription experiments

## Reverse transcription using M-MuLV RT (RNase H$^-$) (T5)

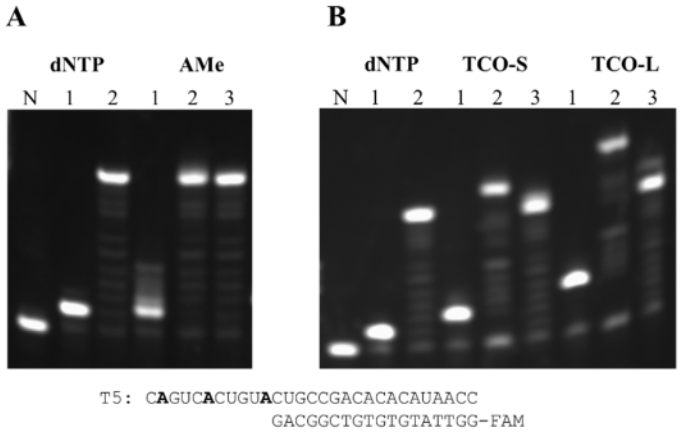



**Figure S-7.** Efficient reverse transcription reactions using M-MuLV (RNase H$^-$) reverse transcriptase with primer P4 and template T5. 3.2 nmol of each triphosphate was used unless otherwise stated. A). Reactions with AM dUTP; B). Reactions with TCO dUTPs. Lane N, primer and template without dNTPs; dNTP lanes, unmodified triphosphate reactions; AM lanes, modified AM dUTP reactions; TCO lanes, modified TCO dUTP reactions; Lane 1, dTTP or modified dUTP; Lane 2, dTTP or modified dUTP + dATP + dCTP + dGTP; Lane 3, dTTP + modified dUTP (1.6 nmol of each) + dATP + dCTP + dGTP (20 % PAGE gel).

These PAGE experiments indicate that M-MuLV RT (RNase H$^-$) reverse transcriptase successfully incorporated AM dUTP and TCO dUTPs into RNA template T5.

## Reverse transcription using AMV reverse transcriptase (T5 and T6)

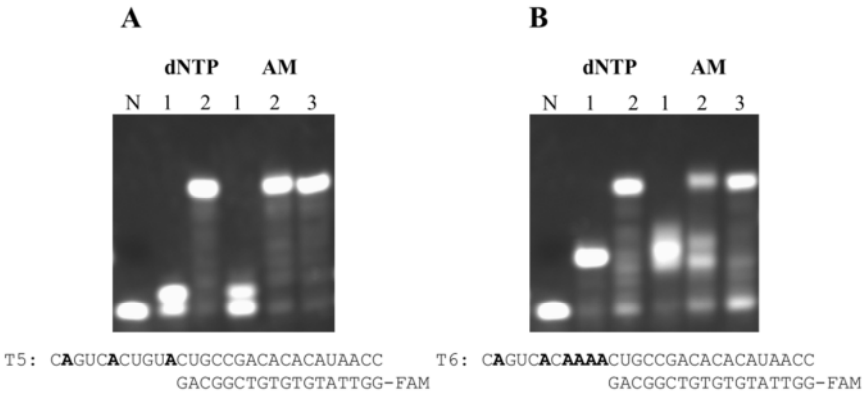



**Figure S-8.** Successful reverse transcription using AM dUTP with AMV reverse transcriptase. 3.2 nmol of each triphosphate was used unless otherwise stated. A). Reaction with primer P4 and template T5; B). Reaction with primer P4 and template T6. Lane N, primer and template without dNTPs; dNTP lanes, unmodified triphosphate reactions; AM lanes, modified AM dUTP reactions; Lane 1, dTTP or modified dUTP; Lane 2, dTTP or modified dUTP + dATP + dCTP + dGTP; Lane 3, dTTP + modified dUTP (1.6 nmol of each) + dATP + dCTP + dGTP (20% PAGE gel).

These PAGE experiments indicate that AMV reverse transcriptase successfully incorporated AM dUTP into RNA template T5 and T6.

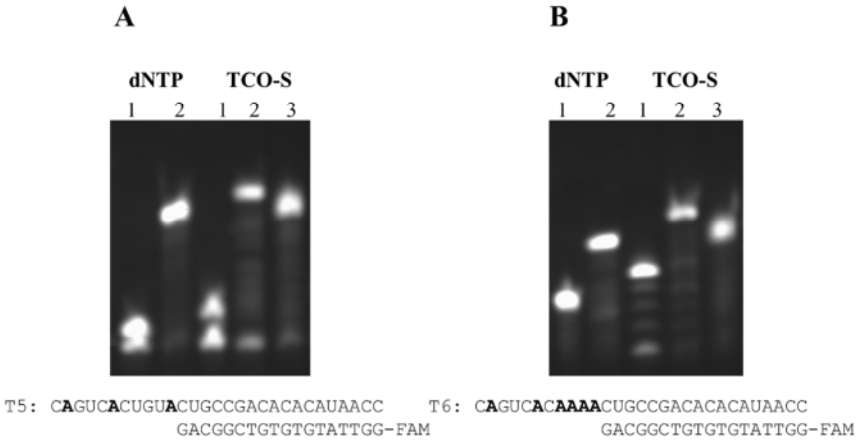



**Figure S-9.** Efficient reverse transcription reactions using AMV reverse transcriptase with TCO-S dUTP. 3.2 nmol of each triphosphate was used unless otherwise stated. A). Reaction with primer P4 and template T5; B). Reaction with primer P4 and template T6. Lane N, primer and template without dNTPs; dNTP lanes, unmodified triphosphate reactions; TCO-S lanes, modified TCO-S dUTP reactions; Lane 1, dTTP or modified dUTP; Lane 2, dTTP or modified dUTP + dATP + dCTP + dGTP; Lane 3, dTTP + modified dUTP (1.6 nmol of each) + dATP + dCTP + dGTP (20% PAGE gel).

These PAGE experiments indicate that AMV reverse transcriptase successfully incorporated TCO-S dUTP into RNA template T5 and T6.

## Characterization of primer extension and reverse transcription products by mass spectrometry

**Table S-1.** Mass data for primer extension reactions

| | T1 | | | | T2 | | | |
|---|---|---|---|---|---|---|---|---|
| | dUTP | | A+C+G+dUTP | | dUTP | | A+G+C+dUTP | |
| | Calc. mass | Found | Calc. mass | Found | Calc. mass | Found | Calc. mass | Found |
| dTTP[all] | 6442 | 6442 | 9556 | 9555 | 7355 | 7355 | 9553 | 9553 |
| AM | 6483 | 6483[G,D,K,M] | 9679 | 9679[G,D,K,M] | 7519 | 7519[G,D,K] | 9799 | 9798[G,D,K] |
| TCO-S[all] | 6633 | 6633 | 10129 | 10129 | 8119 | 8120 | 10699 | 10701 |
| TCO-L | 6880 | 6880[all] | 10870 | 10870[all] | 9108 | 9108[all] | 12181 | 12184[G] |

Note: The superscripts indicate the polymerase that was used to give the extended primers. All = all following five enzymes. G = GoTaq, K = Klenow, D = KOD, M = M-MuLV (RNase $H^-$), AMV reverse transcriptase.

## S5: Melting experiments on polymerase chain reaction products

Melting temperature was measured directly after RT-PCR reactions on a Bio-Rad CFX96 instrument. The reaction mixture was heated to 95 °C for 30 s, cooled to 30 °C, then the temperature was increased at 1 or 0.5 °C per second to 95 °C and held at each temperature for 5 s. PCR reactions and melting experiments were repeated few times and the average melting temperature values were shown in Figures 7 to 10 in the paper.

## Error calculations:

Melting temperature errors were calculated using the confidence intervals derived using Student´s t distribution with 95 % confidence. For $\Delta T_m$ the errors were calculated using the normal error propagation formula without the covariance factor. In graphs these errors are represented by error bars.

## Sequence amplified region of plasmid

```
  501 AAGATATGGG ACATAAACGT TTGGCTTTAG AGGCTGGAGA AGACCCTGTA
  551 AACAATCCTA TTGAATATAT TCTTGACTGT ATCAAAACCA TATACAGCAT
  601 AAAACATAAA AATGGAGCAA TTAGACGTGT AAATGTAAAT ATTGCAGCTA
  651 CTACTGTAGA AAACTACAAG AAATTAAAGG ATGCTGGTAT TGGAACATAT
  701 ATACTTTTCC AAGAAACCTA TAACAAAAAA AGTTACGAGG AACTTCATCC
  751 TACAGGTCCA AAACATGATT ATGCCTATCA TACAGAAGCA ATGGATCGTG
  801 CTATGGAAGG TGGTATTGAT GATGTAGGTA TTGGGGTTTT GTTTGGACTA
  851 AATATGTACA AATATGACTT TGTTGGACTT CTAATGCATG CTGAACACTT
  901 GGAAGCTGCT ATGGGTGTAG GCCCTCATAC TATAAGCGTT CCTCGTATAC
  951 GTCCTGCAGA TGACATTGAT CCTGAAAACT TCTCAAATGC AATATCGGAC
 1001 GAGATTTTTG AAAAAATTGT AGCCATTATT CGTATTGCAG TTCCATACAC
```

**Primer 10** GTTTGGCTTTAGAGGCTGGAG

**Primer 11** ACTGCAATACGAATAATGGCTAC

## Melting experiments with PCR products from template T9

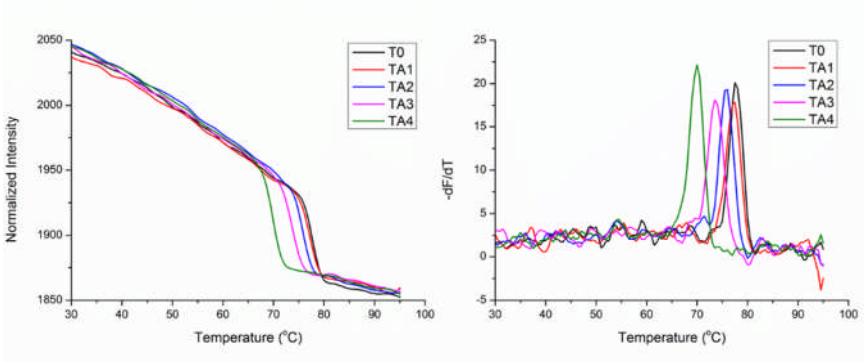



**Figure S-10.** Fluorescence melting curves and derivatives on PCR amplicons with different dTTP and AM dUTPs ratios (5 nmol in total of AM dUTP/dTTP + 5 nmol each of dATP + dCTP + dGTP) using GoTaq polymerase with SYBR Green DNA binding dye. T0, unmodified amplicon; TA1 to TA4 = 25 to 100 % AM dUTP relative to dTTP.

These experiments indicate that AM dUTP supported successful amplification in PCR reactions using GoTaq polymerase and template T9. They also indicate that AM dUTP destabilises the DNA duplex.

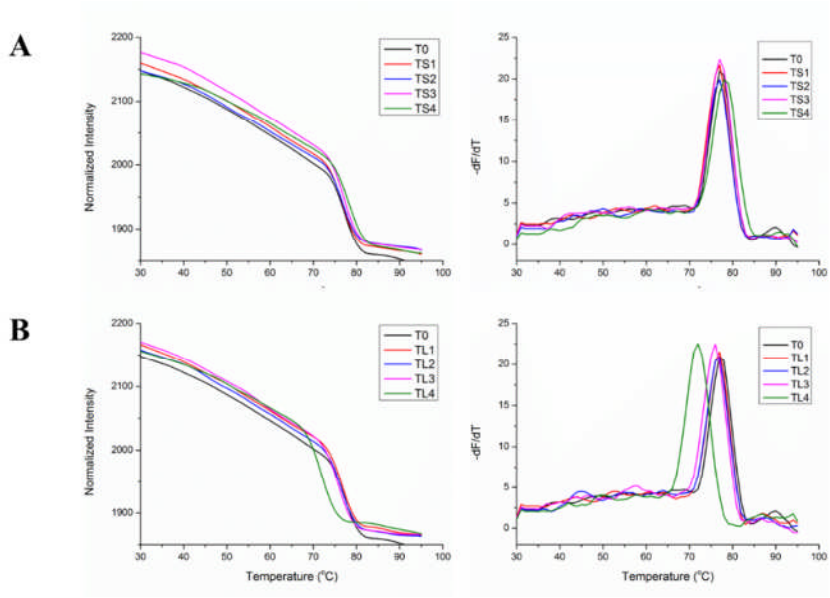



**Figure S-11.** Fluorescence melting curves and derivatives on PCR amplicons with different dTTP and TCO dUTPs ratios (5 nmol in total of TCO dUTP/dTTP + 5 nmol each of dATP + dCTP + dGTP) using GoTaq polymerase with SYBR Green DNA binding dye. T0, unmodified amplicon. A). TS1 to TS4 = 25 to 100 % TCO-S dUTP relative to dTTP; B). TL1 to TL4 = 25 to 100 % TCO-L dUTP relative to dTTP.

These experiments indicate that TCO dUTPs supported successful amplification in PCR reaction using GoTaq polymerase and template T9. TCO-S dUTP maintains DNA duplex stability, whereas TCO-L dUTP destabilises the DNA duplex.

## Melting experiments with PCR products from template T12

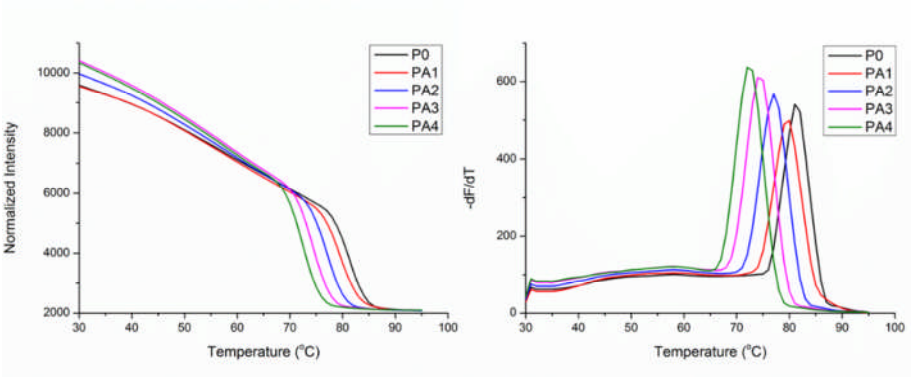



**Figure S-12.** Fluorescence melting curves and derivatives on PCR amplicons with different dTTP and AM dUTP ratios (10 nmol in total of AM dUTP/dTTP + 10 nmol each of dATP + dCTP + dGTP) using KOD polymerase with SYBR Green DNA binding dye. P0, unmodified amplicon; PA1 to PA4 = 25 to 100 % AM dUTP relative to dTTP.

These experiments indicate that AM dUTP supported successful amplification in PCR reaction using KOD polymerase and plasmid template T12. AM dUTP destabilises the DNA duplex.

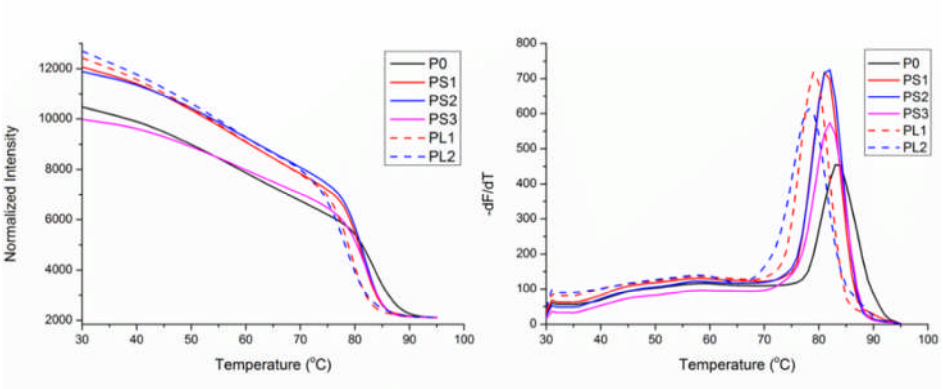



**Figure S-13.** Fluorescence melting curves and derivatives on PCR amplicons with different dTTP and TCO dUTP ratios (10 nmol in total of TCO dUTP/dTTP + 10 nmol each of dATP + dCTP + dGTP) using KOD polymerase with SYBR Green DNA binding dye. P0, unmodified amplicon; PS1 to PS3 = 25 to 75 % of TCO-S dUTP relative to dTTP. PL1 to PL2 = 25 %, 50 % of TCO-L dUTP relative to dTTP.

These experiments indicate that TCO dUTPs supported successful amplification in PCR reactions with different percentages of dTTP and TCO dUTPs using KOD polymerase and

plasmid template T12. TCO-S dUTP maintains duplex stability whereas TCO-L dUTP destabilises the DNA duplex.

## S6: Fluorescent labelling of TCO-S dUTP PCR products from templateT9

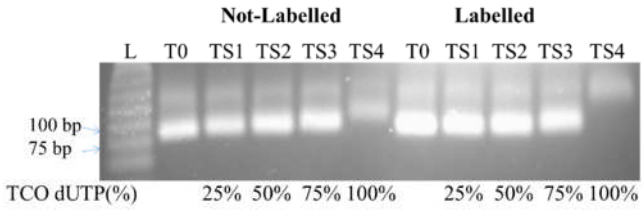



**Figure S-14.** Fluorescence labelling on TCO dUTP PCR amplicons from template T9 with 6-methyl-tetrazine-sulfo-Cy3 for 30 min. TS1 to TS4 = 25 to 100% of TCO-S dUTP relative to dTTP. (2 % Agarose gel, stained with ethidium bromide)

These experiments indicate that TCO dUTP PCR amplicons were efficiently labelled with 6-methyl-tetrazine-sulfo-Cy3 in 30 min. The labelled products exhibited lower electrophoretic mobility.

## Reference:

1. I. S. Hong, H. Ding and M. M. Greenberg, *J. Am. Chem. Soc.* , 2006, 128, 485-491.
2. X. Ren, M. Gerowska, A. H. El-Sagheer and T. Brown, *Bioorg. Med. Chem.*, 2014, 22, 4384-4390.