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Fluorogenic Sequencing Using Halogen-Fluorescein-Labeled Nucleotides

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Fluorogenic sequencing is a sequencing-by-synthesis technology that combines the advantages of pyrosequencing and fluorescence detection. With native duplex DNA as the major product, we employ polymerase to incorporate the complementarily matched terminal phosphate-labeled fluorogenic nucleotides into the DNA template and release halogen-fluorescein as the reporter. This red-emitting fluorophore successfully avoids spectral overlap with the autofluorescence background of the flow chip. We fully characterized the enzymatic reaction kinetics of the new substrates, and performed a 35-base sequencing experiment with 60 reaction cycles. Our achievement expands the substrate repertoire for fluorogenic sequencing, and extends the spectral range to obtain better signal-to-background performance.

Next-generation sequencing (NGS) technologies have revolutionized genomic studies[1] by providing massive DNA sequences with high throughput at a cost that is over four orders of magnitude lower than that of the previous-generation Sanger sequencing.[2–6] The majority of NGS technologies can be divided into two groups: sequencing by synthesis (SBS) and sequencing by ligation (SBL).[2,7] Because of the low efficiency of ligase and the complexity of the SBL workflow, SBS has become the dominant strategy. SBS technologies obtain the DNA sequences by measuring specific optical or electrical signals coupled with complimentary-strand synthesis by a polymerase. For SBS, there are two major approaches: reversible terminator sequencing[3,7–14] and pyrosequencing.[3,15,16] In reversible terminator sequencing, four different nucleotides (with the 3′-OH blocked by a reversible terminator group and a cleavable fluorescent dye conjugated to the base) are presented to the target template simultaneously, and one of the labeled nucleotides is incorporated.[17] The incorporated nucleotides can later be determined by optical imaging, and their different colors identify the bases. A potential limitation is that after the fluorescence labels are cleaved, a "molecular scar" is left in the DNA molecule, and accumulation of these scars can eventually hinder the stability of the duplex DNA and thus limit sequencing length.[18]

In contrast, pyrosequencing measures the byproducts of the DNA synthesis process.[19] The target for detection can be either pyrophosphate, which can trigger a bioluminescence cascade[15,20,21] or be detected directly by chelator probes,[22,23] or the proton that is released through nucleotide incorporation.[24,25] The advantages of pyrosequencing are that native nucleotides are used, so the final duplex DNA products are natural molecules, thus ensuring a fast reaction and long-sequence reads.[16] One technical limitation of pyrosequencing is that the detection has to be continuous because the signals are transient.[19] To overcome this limitation, fluorogenic dyes labeled on the terminal phosphate of the nucleoside triphosphate (terminal phosphate-labeled fluorogenic nucleotides, TPLFNs) are used as reporter molecules in DNA polymerization.[26] We have previously shown that certain DNA polymerases can recognize TPLFNs as natural dNTPs, thus incorporating the matched (correct Watson–Crick pairing) fluorogenic bases onto the DNA template with annealed primers (rejecting mismatched TPLFNs), thereby affording sequencing information.[27] By employing fluorescence detection and a native DNA product, this fluorogenic SBS method shows great potential, by combining the long read-length of pyrosequencing and the accurate measurement of accumulated fluorophores. This allows not only scalable throughput but also significantly improved homopolymer quantification. Our previous design employed 3′-*O*-methyl-5(6)-carboxyfluorescein (FAM-OMe) as the reporter fluorophore (λ_{em} = 520 nm, λ_{ex} = 473 nm). However, FAM-OMe has a relatively broad emission spectrum and low quantum yield (Figure 2B and C, below), so requires powerful excitation to yield sufficient fluorescence for detection. Furthermore, we found that under these excitation/detection conditions much of the material used for flow-cell construction contributed faint background autofluorescence, which greatly affected the fluorogenic measurement. A straightforward approach is to develop fluorophores with longer excitation and emission wavelengths for TPLFNs.

Each fluorogenic sequencing cycle has two steps (Figure 1A). First, the primers annealed on the template are extended by DNA polymerase by incorporation of complemen-

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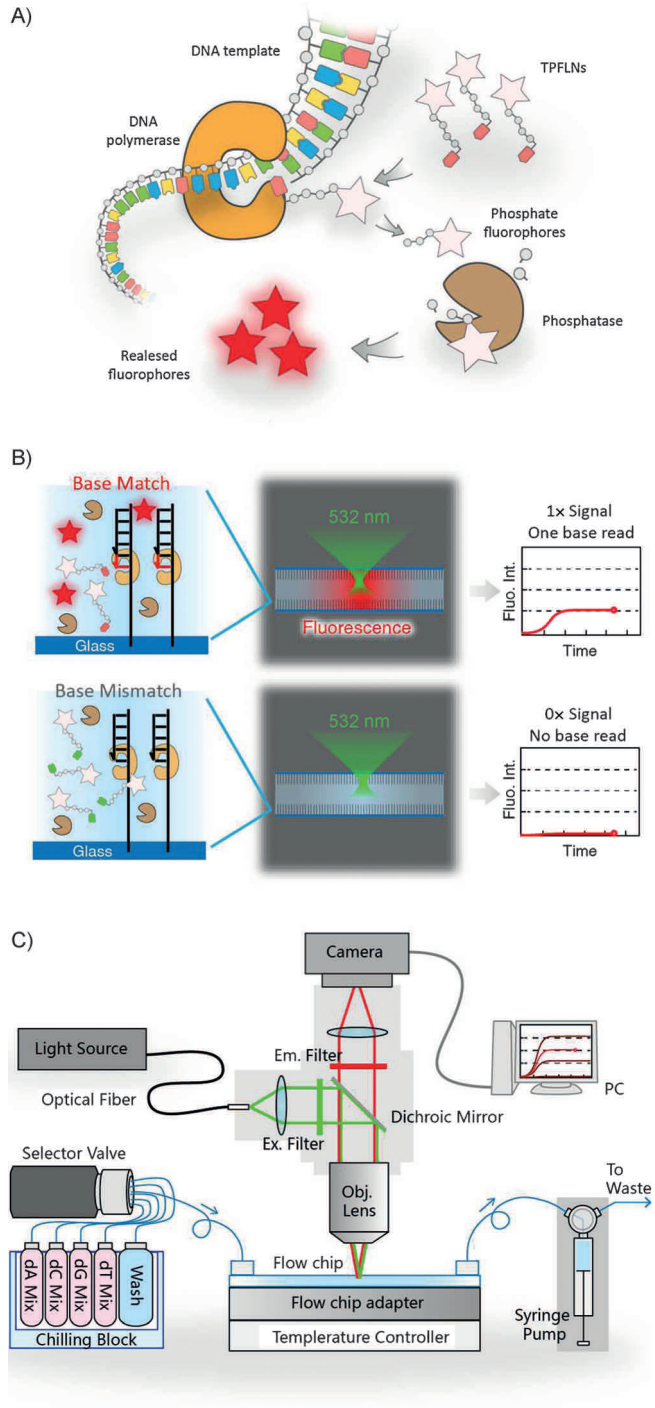


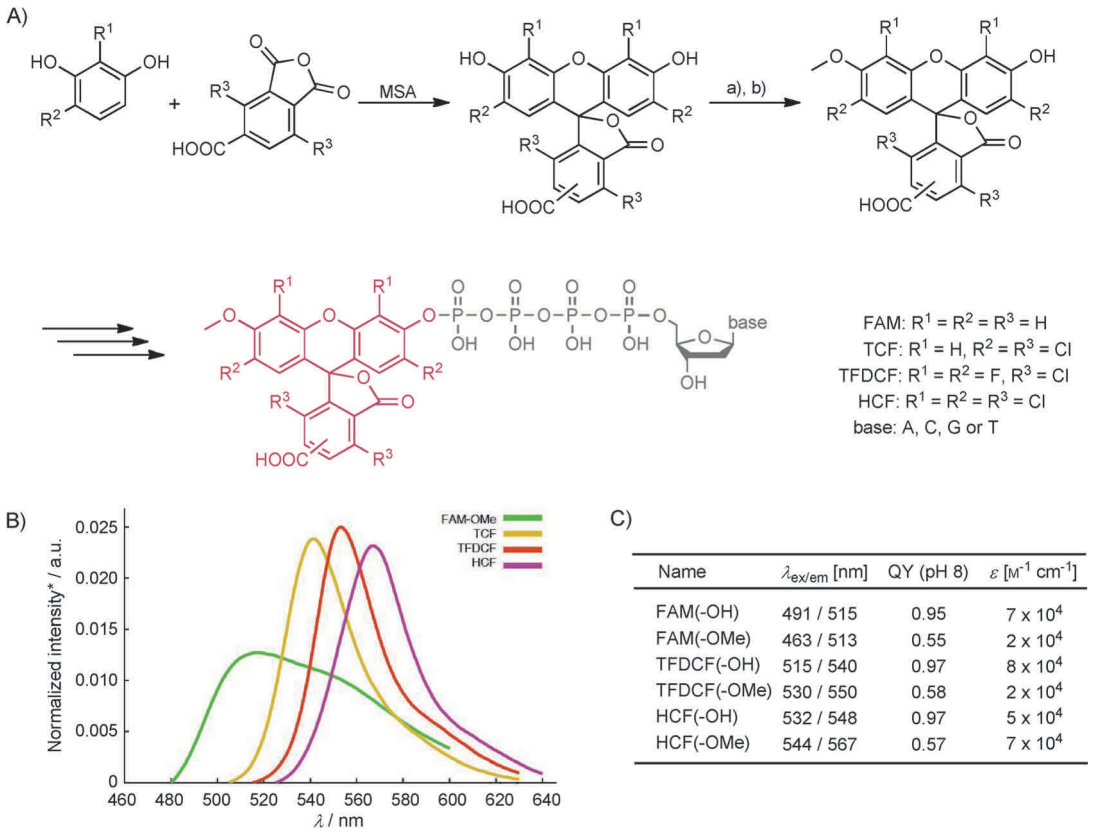
Figure 1. Fluorogenic sequencing process and sequencer construction. A) Non-fluorescent TPFLNs release fluorophores upon incorporation by polymerase and phosphate digestion by phosphatase. B) Signal observation of each reaction cycle. ssDNA template is immobilized on the surface of a flow cell. Matching TPFLNs extend the annealed primers and generate fluorophores. The fluorescence intensity rises to a plateau. No primer extension takes place with mismatched TPFLNs, and the fluorescence intensity does not increase. C) Schematic illustratoin of a sequencer. The flowchip is mounted on a temperature controller, with one port connected to a selector valve to sequentially select specific reagents stored in the chilling block. The other port of the flow chip is connected to a syringe pump, which draws reagent or wash buffer from the flowchip. An epi-illuminated microscope module with a highly sensitive sCMOS camera collects the data.

tarily matched TPLFNs, thereby releasing the nonfluorescent polyphosphate dyes into solution. The polyphosphate chains of the released molecules are rapidly digested by alkaline phosphatase, thus yielding active fluorophores. The fluorescence signal is used to differentiate the matched base from mismatched ones. When the different TPLFNs (dA, dT, dG, and dC) are individually introduced consecutively in the reaction, we can sequence the template DNA by measuring the fluorescence of each cycle.

DNA templates (with annealed primers) are immobilized on a solid surface, and the reagents (*Bst* polymerase, alkaline phosphatase (CIP), and TPFLNs) flow over the surface in each cycle (Figure 1 B). Between cycles, the residue of the previous reaction needs to be thoroughly washed. Given sufficient reaction time, the matched TPFLNs release fluorophores with a yield proportional to the number of homopolymeric complementary bases. Although, ideally, the mismatched TPFLNs will not generate fluorophores, a weak fluorescence signal might still be observed with long incubation due to misincorporation and intrinsic hydrolysis of TPFLNs.

To test the fluorogenic sequencing performance of the newly developed TPFLNs containing red-emitting fluorophores, we constructed a single-chamber sequencer to carry out the automatic reaction cycles (Figure 1 C). All the reagents were stored at 4 °C before introducing to the reaction chamber in the flow chip, which was mounted on a Peltier temperate controller. A selector valve sequentially introduced individual reaction components (a specific TPFLN with CIP and *Bst* in *Bst* reaction buffer) to the chip, as well as the wash buffer between different TPFLNs. The sequencing reaction of each cycle was performed at 65 °C (optimum for *Bst* polymerase); the wash step was performed at 4 °C to prevent loss of the immobilized template or the annealed primers. The fluorescence signal was acquired by an epi-illuminated microscope module (fiber-coupled LED light source, set of filters/dichroic mirror, high numeric aperture objective lens, and a highly sensitive sCMOS camera). In order to investigate the dynamic behavior of fluorogenic incorporation of TPFLNs, we continuously monitored the fluorescence signal from the flow chip at 1 Hz.

An ideal TPFLN substrate should be unambiguously recognized by the DNA polymerase, emit a low background signal in the nonfluorescent state, and exhibit a superior on/off ratio of fluorescence intensity when the fluorophore is released by phosphatase digestion. One of our major goals was to develop a fluorogenic molecule with longer working wavelengths than those of FAM-OMe.[27] We developed a concise synthesis process to obtain a series of halogen-modified fluorescein derivatives (Figure 2 A). Halogen substituents, which have been shown to affect absorption and emission wavelengths, can be readily introduced into the ring system of fluorescein by a convenient one-step condensation of halogenated resorcinol and phthalic anhydride derivatives. Thus, 2′,4′,5′,7′-tetrachloro-5(6)-carboxyfluorescein (TCF), 2′,4′,5′,7′-tetrafluoro-4,7-dichloro-5(6)-carboxyfluorescein (TFDCF), and 2′,4,4′,5′,7,7′-hexachloro-5(6)-carboxyfluorescein (HCF) were synthesized by using the appropriate materials, with methanesulfonic acid as solvent, Brøn-



Name	$\lambda_{ex/em}$ [nm]	QY (pH 8)	ε [M^{-1} cm^{-1}]
FAM(-OH)	491 / 515	0.95	7 x 10^4
FAM(-OMe)	463 / 513	0.55	2 x 10^4
TFDCF(-OH)	515 / 540	0.97	8 x 10^4
TFDCF(-OMe)	530 / 550	0.58	2 x 10^4
HCF(-OH)	532 / 548	0.97	5 x 10^4
HCF(-OMe)	544 / 567	0.57	7 x 10^4

Figure 2. Halogen–fluoresceins. A) Synthetic route to halogen–fluorescein, and structures of TPLFNs. a) MeI/Base; b) OH^-/MeOH. B) Fluorescence spectra of halogen–fluoresceins. C) Photophysical parameters of the fluorophores.

sted acid catalyst, and heating (see Section 1 in the Supporting Information).[26,28–30]

One lesson we learned was that the 3′-O-methylation of fluorescein chromophore is essential for the fluorogenic property. This modification inevitably lowers the quantum yield of the fluorescein moiety but has a negligible effect on the absorption and emission spectra. As expected, the halogen-modified derivatives clearly red-shift the absorption and emission spectra (Figure 2B and C). Notably, all these halogen–fluorescein compounds have narrower emission bands, thus making fluorescence detection over a given spectral range more efficient. To avoid spectral overlap with commonly used flow-chip material (glass and polydimethylsiloxane), we chose 3′-O-methylated HCF as the fluorophore for the synthesized TPFLNs and studied their sequencing performance.

We first investigated the reaction activity and kinetics of the HCF-based TPFLNs. Polyphosphate digestion is over two orders of magnitude faster than polymerization; hence once a TPFLN is incorporated by the polymerase the released phosphate dye molecules are turned into free dye almost immediately. The intensity of the measured fluorescence quantitatively reflects the total amount of incorporated TPFLN.

We developed a fluorogenic measurement device (Figure S2.1 in the Supporting Information) to monitor the long-term kinetics of DNA polymerization with TPFLNs. This device allows simultaneous multiple fluorescent reactions (5–20 μL) in a single 6×8-well plate. Thus, we carried out combinatorial single-base-extension experiments to test the pairing performance of the HCF-TPFLNs (Figure 3A). We placed 1 pmol of pre-annealed DNA template–primer (single-base overhangs) into each microwell, arranged as four groups according to the different overhanging bases. Then an excess of HCF-TPFLNs (dA, dT, dG, and dC; 10 pmol) was added, thus forming 16 combinatorial reactions (each in duplicate). Next, pre-cooled reaction master mix (Supporting Information) was added to each well, followed by gentle mixing. Finally, the plate was sealed, and the temperature was raised from 4 to 65 °C to initiate the reactions. All four complementary combinations (dA with T overhang, dT with A, dC with G, and dG with C) exhibited strong fluorescence, whereas the other combinations produced very weak fluorescence (principally a consequence of misincorporation and nonspecific hydrolysis). Furthermore, the four positive combinations showed similar reaction rates, thus indicating that *Bst* polymerase did not differentially recognize the four HCF-TPFLNs when incorporating matched nucleotides, hence reducing the technical complexity of the sequencing operation.

We next tested whether misincorporation of mismatched HCF-TPFLNs compromises sequencing performance. We designed a looped ssDNA-free extension experiment to assess the effective elongation length of *Bst* polymerase with HCF-TPFLNs as substrates. *Bst* polymerase has strong strand-displacement activity but lacks a proofreading function. We used a commercially available 7249-base template isolated from

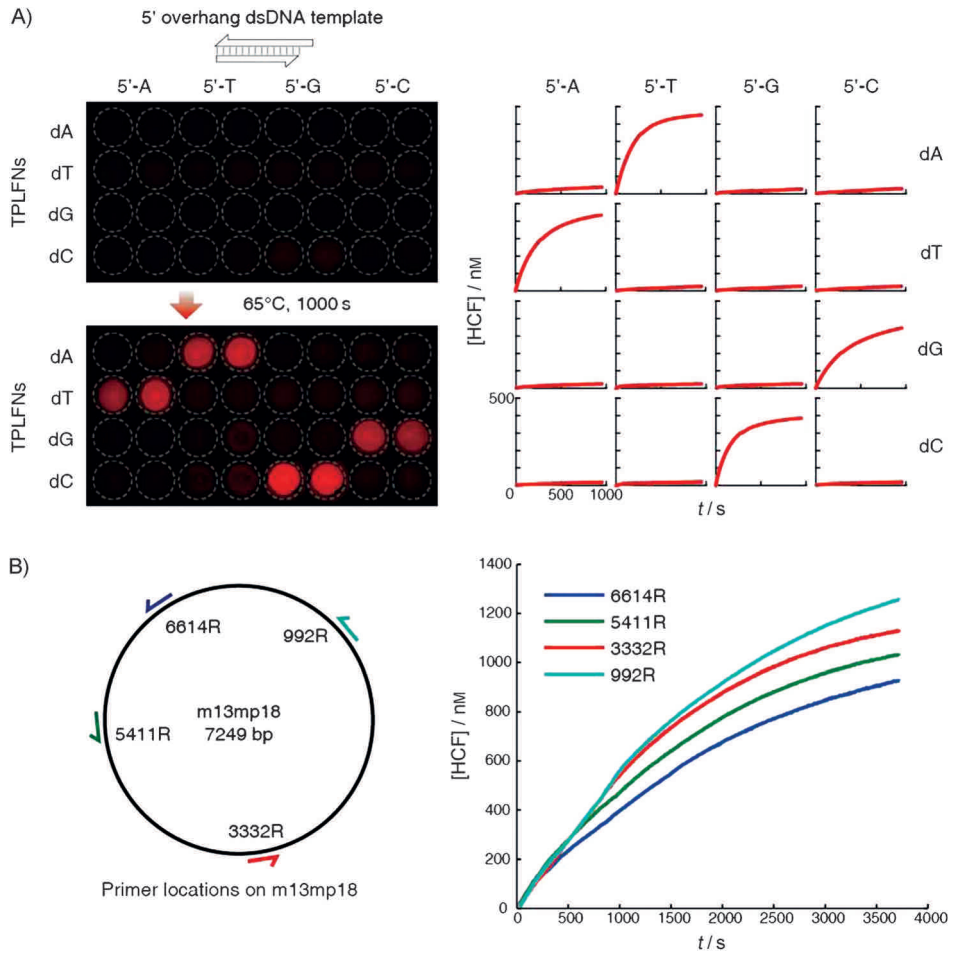


Figure 3. DNA polymerization using TPLFNs in a single-base extension experiment. A) Fluorescence images taken before (top) and after (below) TPLFN reaction with 5′-overhang template. Plots of concentration rise show similar reaction rates for all correct combinations. B) DNA template and primers used for testing misincorporation. Each primer elongates until misincorporation, thereby resulting in decreased reaction rate.

M13mp18 bacteriophage (Figure 3B). We synthesized four fully complementary primers, and these (added in excess: final concentration 0.5 nM) were annealed to the template, followed by addition of pre-cooled HCF-TPFLN reaction master mix. The reactions were activated by heating (65 °C), and the elongation process was monitored fluorescently. Each incorporation event released a fluorescent molecule, and elongation proceeded until a misincorporation, which prevented further elongation. We observed that all reactions eventually slowed and produced ~1 μM HCF in the solution, thus indicating that on average elongation could achieve at least 2000 bases, which is sufficient for practical sequencing applications.

Finally, we tested the fluorogenic sequencing performance of HCF-TPFLNs with *Bst* polymerase on our single-tube sequencer setup. ssDNA sequencing templates were generated by solid-phase PCR (~2 fmol mm^{-2}),[31–33] with the 5′-ends immobilized on the glass surface of a flow chip (sequencing details are given in the Supporting Information). Sequencing primers (33 bases) were then annealed to the templates. Each 4 min sequencing cycle comprised a 90 s wash step, a 90 s reaction step (65 °C), and a 60 s imaging step (15 °C), with additional time for reagent switching and temperature ramping. The fluorescent signal from the reaction chamber was recorded every 1 s for each reaction cycle (Figure 4A). For reaction cycles in which TPFLNs were properly incorporated onto the complementary bases of the template, the fluorescence intensity significantly increased with the rise in temperature. The fluorogenic signals were calculated from these fluorescence time traces (Figure 4B). On average, we observed a 1.7% decay of single-base fluorescence intensity between adjacent reaction cycles. We also observed a continuous fluorescence increase (~10 ppm s^{-1}), mainly as a result of TPLFN hydrolysis. This slow hydrolysis generated a negligible (~0.1% relative to substrate) fluorescence background during a typical reaction cycle. This intensity decay probably had two causes: 1) loss of the immobilized template or primer during the wash step, and 2) termination of nucleotide extension for a small portion of primers after misincorporation. Assuming signal degeneration is constant across cycles, the fluorescence intensity of each reaction cycle can be corrected by an exponential decay function. Corrected fluorescence intensity (i.e., fluorophore concentration) directly reflects the homopolymeric number of each corresponding base (Figure 4C). This base-detecting process can reveal the sequence of the template DNA. This HCF–TPFLN-based fluorogenic sequencing method obtained a 35-base sequence in a 60-cycle run without errors.

In summary, with hexachlorocarboxyfluorescein as a new fluorogenic moiety, terminal-phosphate-labeled fluorogenic nucleotides can be unambiguously recognized and incorporated by *Bst* polymerase in order to sequence ssDNA. Halogen substitution successfully red-shifted fluorescein emission to 567 nm, thereby eliminating spectral overlap between the fluorogenic emission and the background fluorescence of the sequencing chip. Our high-performance single-tube sequencer using these red-emitting nucleotides has expanded the substrate repertoire of fluorogenic sequencing, and makes multicolor fluorogenic sequencing-by-synthesis technology an attractive approach in the future.

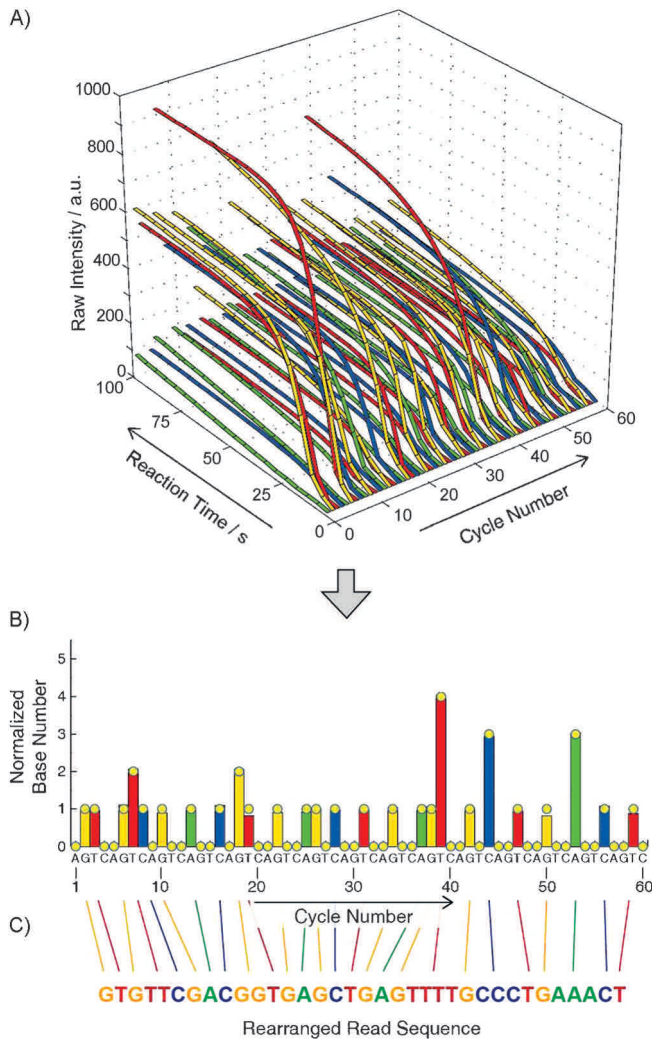


Figure 4. Fluorogenic sequencing using halogen–fluorescein TPFLNs. A) Fluorescence kinetics of 60 sequencing cycles. B) Normalized signals are extracted from the fluorescence kinetic curves and converted into base number. C) The DNA sequence is obtained from rearranged normalized base-number sequence.

Acknowledgements

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Keywords: DNA · fluorescent probes · fluorogenic sequencing · sequencer · sequencing by synthesis · phosphate-labeled nucleotides

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Supporting Information

Fluorogenic Sequencing Using Halogen-Fluorescein-Labeled Nucleotides

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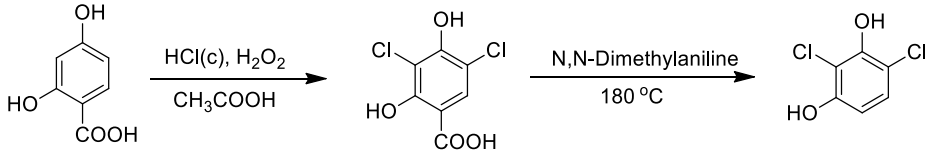
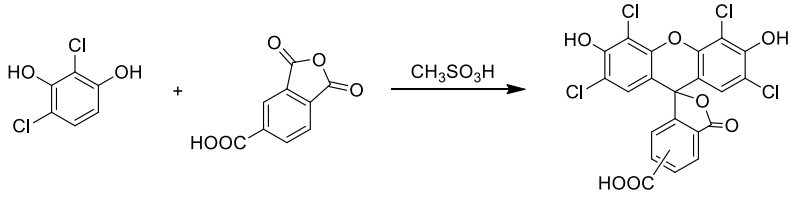
Section 1. Dye Synthesis and Characteristics

General procedures and methods: Anhydrous solvents were freshly distilled using general procedure (Na or CaH_2). Reagents were used as received from commercial suppliers unless otherwise stated. Air- and/or moisture-sensitive experiments were carried out under an atmosphere of Argon. The progress of reaction was monitored by analytical thin layer chromatography (TLC) using Merck Silica gel 60 F254 plate. The plates were visualized under UV-light at λ =254 nm or 360 nm. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avnce-III 500 MHz spectrometer. Chemical shifts are reported relative to residual solvent peaks. Steady state fluorescence spectra were recorded using a spetrafluoremeter (Fluorolog-3, Horiba Jobin-Yvon). Mass spectral analyses were carried out with Bruker APEX IV Mass Spectrometer and AB Sciex MALDI-TOF 5800 Spectrometer. Reverse phase HPLC was carried out on a Shimadzu LC-20A HPLC system. Sample was dissolved in water and analyzed by analytical Inertsil ODS-3 C18 column (250 x 4.6 mm, 5μm) at 1mL/min flow rate, with a gradient of B (CH_3CN) in A (50 mM TEAA pH 7.3) (0-20% of B over 15 min, 20-30% of B over 10 min).

I. Halogen-fluorescein Synthesis:

1) Synthesis of 2′,4′,5′,7′-tetrachloro-5(6)-carboxyfluorescein (**TCF**)





2,4-dichlororesorcinol is synthesized using the following procedure:

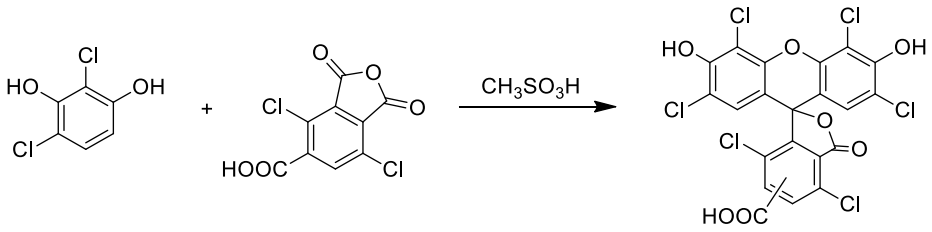
To a 500 mL two-neck round bottom flask equipped with a dropping funnel, 200 mL acetic acid and 25 g (0.16 mol) 4-Carboxy resorcinol were mixed together. To this mixture 30 mL hydrochloric acid (concentrated) was added and the mixture was heated to 65 ^{o}C with stirring till the suspension solution become clear. Then 50 mL 30% hydro peroxide was added

slowly through dropping funnel within 2 h. After that the reaction mixture was stirred at 65 ^{o}C for 2 h before cooled to 4 ^{o}C. The precipitated solid was filtered and washed with cooled water. The collected solid was recrystallized in 2:1 (v/v) H_2O/ethanol to obtained 30 g (82% yield) of 3,5-dichloro-2,4-dihydroxybenzoic acid as white solid.

The 3,5-dichloro-2,4-dihydroxybenzoic acid (12.0 g) was suspended in 40 mL N,N-dimethyl aniline, the mixture was degassed and slowly heated to 185-195 ^{o}C under Ar(g). After stirring 3 h at the same temperature the reaction mixture was cooled to rt and the brown solution was poured into 50 mL conc. HCl at 0 ^{o}C with rapid stirring. The mixture was extracted with CH_2Cl_2 (40 mL x 5), then the combined organic phase was washed with 6 N HCl and brine and dried by $MgSO_4$. Solvents were removed in vacuo to give a yellow oil. Purification by silica flash column chromatography to afford 2,4-dichlororesorcinol in 65% yield as white solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.51 (s, 1H), 5.84 (s, 1H), 5.58-6.66 (d, 1H), 7.15-7.17 (d, 1H).

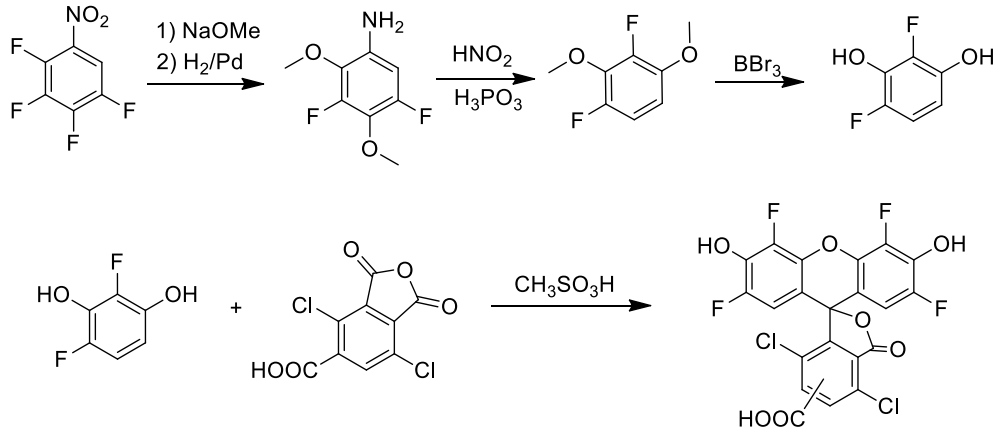
To synthesis **TCF**, 3.76 g (21.0 mmol) of the above synthesized 2,4-dichlororesorcinol and 1.92 g (10.0 mmol) of trimallitic anhydride (from J&K Chemical) was mixed in a flame dried 250 mL round-bottom flask with 60 mL of methanesulfonic acid. The mixture was heated for 3 h at 120 ^{o}C, then 20 h at 150 ^{o}C under Ar(g). Then the dark red mixture was cooled and poured slowly into 200 mL of rapidly stirred ice water. The precipitated solid was collected by suction filtration and washed with 200 mL of cold water, and dried by pump to afford the crude product **TCF**. Further purification can be performed by silica gel flash chromatography to give brown-red solid in 47% yield. HRMS (ESI): M-1: 510.8933 (calc 510.8951 for $C_{21}H_7Cl_4O_7$). ^{1}H NMR (500 MHz, DMSO) δ 8.63 (s, 1H, Ar-H), 8.04 (s, 1H, Ar-H), 8.00 (d, *J* = 10Hz, 1H, Ar-H), 7.61 (s, 1H, Ar-H), 7.07 (d, *J* = 10Hz, 1H, Ar-H), 6.79 (s, 2H, Ar-H,), 6.78 (s, 2H, Ar-H). ^{13}C NMR (126 MHz, DMSO) δ 170.60, 170.12, 169.88, 169.76, 168.07, 158.65, 158.63, 151.69, 151.64, 141.25, 140.97, 140.46, 140.20, 133.70, 132.16, 131.70, 130.12, 129.96, 129.79, 129.57, 128.34, 126.70, 126.58, 108.69, 108.60.

2) Synthesis of 2',4',5',7',4,7-hexachloro-5(6)-carboxyfluorescein(HCF) (**HCF**)



3,6-dichloro-trimallitic anhydride is prepared according to the reported method[1]. **HCF** was synthesized with the similar procedure as above for **TCF**. After condensation reaction in methanesulfonic acid, the desire product HCF was obtained in 40% yield as dark-red solid. HRMS (ESI): M-1: 578.8150 (calc 578.8161 for $C_{21}H_5Cl_6O_7$). ^{1}H NMR (500 MHz, DMSO) δ 7.71 (s, 1H-Ar, 5-isomer), 7.54 (s, 1H-Ar, 6-isomer), 6.86 (s, 2H-Ar, 6-isomer), 6.84 (s, 2H-Ar, 5-isomer). ^{13}C NMR (126 MHz, DMSO) δ 168.52, 168.48, 168.00, 151.71, 148.03, 130.97, 130.32, 129.34, 127.71, 127.44, 127.35, 127.30, 126.57, 126.50, 108.84, 108.80, 108.40, 108.35.

3) Synthesis of 2',4',5',7'-tetrafluoro-4,7-dichloro-5(6)-carboxyfluorescein (**TFDCF**)



2,4-difluororesorcinol is synthesized following the reported procedure[2] with slight modification as described below:

1,2,3,4-tetrafluoro-5-nitrobenzene (24g, 123 mmol) was dissolved in 100 ml anhydrous methanol in a 500 mL flask. This solution was cooled to 4 ^{o}C and 30% sodium methoxide (271 mmol) solution in methanol was slowly added with stirring under Ar(g). The reaction was allowed to reach rt within 4~6 hours, monitored by TLC. After starting material was all consumed, the mixture was cooled, quenched with citric acid aqueous solution (1M,

15 mL). Methanol was removed by vacuum evaporator. The residue was extracted with Et_2O (100 mL x2), washed with citric acid aqueous solution and brine, dried over Na_2SO_4, concentrated and purified by silica gel flash column to give 26 g (96%) 1,3-difluoro-2,4-dimethoxy-5-nitrobenzene as yellowish oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.52 (dd, *J* = 2.3, 11.0 Hz, 1H), 4.12 (t, *J* = 1.8 Hz, 3H), 4.00 (d, *J* = 1.2 Hz, 3H)

1,3-difluoro- 2,4-dimethoxy-5-nitrobenzene (26g, 118 mmol) was dissolved in 150 mL ethyl acetate, 10% Pd on activated carbon (2.65 g) was added and the flask was purged with H_2 by putting a long needle connected with a H_2 balloon below the reaction mixture surface. The reaction was stirred overnight at rt and filtered through a short celite padded filter to remove Pd/C. The filtration was concentrated to get the desired product 3,5-difluoro-2,4-dimethoxyaniline in quantitative yield as an oil, which is pure enough for next step reaction. MS (ESI): M+1= 190.10 (calc 189.06).

3,5-difluoro-2,4-dimethoxyaniline (15g, 79 mmol) was added into a 500 mL flask containing 260 mL 4 N hydrochloride acid at 4 ^{o}C. Then cooled 30% sodium nitrite aqueous solution (83 mmol) was added in with stirring. After 1h stirring, a solution of hypophosphorous acid (200 mL, 1.58 mol, 50 wt % in water) was slowly added and the reaction was stirred overnight at 4 ^{o}C under Ar(g). The mixture was warmed to rt for further 2 h and extracted with CH_2Cl_2 (100 mL x3), washed with brine, dried over Na_2SO_4, concentrated and purified by silica gel flash column to give 12 g (90%) 1,3-difluoro-2,4-dimethoxybenzene as yellowish oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.78 (m, 1H), 6.57 (dt, *J* = 4.7 Hz, 9.0 Hz, 1H), 4.01 (s, 3H). 3.84−3.85 (m, 3H).

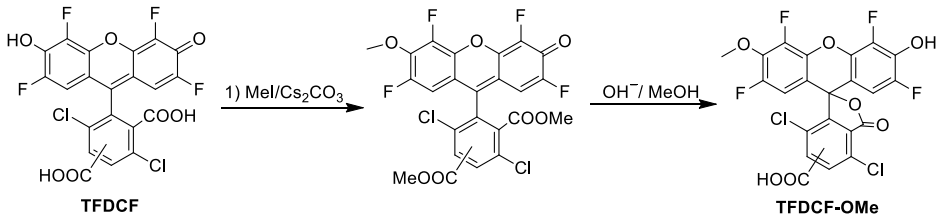
1,3-difluoro-2,4-dimethoxybenzene (12g, 69 mmol) was dissolved in 60 mL anhydrous CH_2Cl_2 at 4 ^{o}C. To this solution was added BBr_3 (1M in CH_2Cl_2, 207 mmol) and the mixture was monitored by TLC. After 15 h, another portion of BBr_3 (69 mmol) was added to ensure the starting material was consumed completely. Then this reaction mixture was poured onto crashed ice and the two phases was separated by separating funnel. The aqueous phase was extracted with CH_2Cl_2 and the combined organic phase was washed with brine, dried over Na_2SO_4, concentrated and purified by silica gel flash column to give 9.4 g (93% yield) of targeted 2,4-difluororesorcinol as light brownish solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.79 (dt, *J* = 2.4 Hz, 9.4 Hz, 1H), 6.50 (dt, *J* = 5.0 Hz, 9.1 Hz, 1H), 5.21 (s, 1H), 4.95 (s,

1H).

To synthesis **TFDCF**, 6.9 g (47.3 mmol) of the above synthesized 2,4-difluororesorcinol and 5.61 g (21.5 mmol) of 3,6-dichloro-trimallitic anhydride was mixed in a flame dried 100 mL round-bottom flask with 50 mL of methanesulfonic acid. The mixture was heated for 5h at 60 ^{o}C and 20h at 90 ^{o}C under Ar(g). Then the dark red mixture was cooled and poured slowly into 200 mL of rapidly stirred ice water. The precipitated solid was collected by suction filtration and washed with 200 mL of cold water, and dried by pump to afford the crude product **TFDCF**. Further purification was performed by silica gel flash chromatography (CH_2Cl_2/MeOH) to give brown-red solid in 47% yield. HRMS (ESI): M+1: 514.9354 (calc 514.9353 for $C_{21}H_5Cl_2F_4O_7$). ^{1}H NMR (500 MHz, DMSO) δ 7.72 (s, 1H, isomer), 7.68 (s, 1H, isomer), 6.45-6.37 (m, 2H, mixture) 6.33 (d, *J* = 10 Hz, 1H, isomer), 6.20 (d, *J* = 10 Hz, 1H,isomer).

II. Halogen-fluorescein 3′-O-Methylation:

1) preparation of **3′-O-methyl-TFDCF (TFDCF-OMe)**



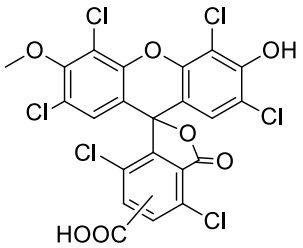
Previous reported method for preparing 3′-O-methyl-FAM (**FAM-OMe**)[3] consisted of three steps, which included acid catalyzed esterification, Methyl iodide methylation and hydrolysis reaction. Here we found that the first two steps can be combined into one by using excessive amount of Methyl iodide to obtain the same intermediate product. The procedure was describe as below:

TFDCF (1.5 g, 2.9 mmol) was put into a 250 mL round-bottom flask containing 30 mL anhydrous DMF and 4.7 g (14.6 mmol) cesium carbonate. The mixture was added MeI (2.8 g, 20 mmol) and stirred overnight at room temperature. DMF was removed by vacuum pump. The residue was extracted with CH_2Cl_2, then washed with 2N HCl and brine, and dried over magnesium sulfate. The organic phase was concentrated to afford the crude intermediate 3′-O-Methylation and esterification compound, which was dissolved in methanol (60 mL) for

next step synthesis without further purification. MS (ESI): M+1: 559.05 (calc 558.99).

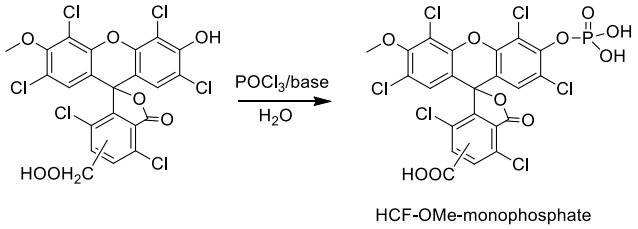
To the methanol solution, 2N LiOH (20 mL in water) was added and the mixture was stirred for 5 h at room temperature. The reaction was monitored by TLC to make sure all starting material was consumed. Then methanol was evaporated and the aqueous residue was acidified with 2N HCl. The resulting precipitate was collected by filtration and dried to afford compound **TFDCF-OMe** (70%), which can be further purified by silica gel chromatograph. HRMS (ESI): M+1: 530.9658 (calc 530.9656 for $C_{22}H_9Cl_2F_4O_7$). ^{1}H NMR (500 MHz, DMSO) δ 7.86 (s, 1H-Ar, 5-isomer), 7.80 (s, 1H-Ar, 6-isomer), 6.38 (d, *J* = 10Hz, 2H-Ar, 5-isomer), 6.35 (d, *J* = 15Hz, 2H-Ar, 6-isomer), 3.46 (s, 3H, CH_3, mixture).^{13}C NMR (126 MHz, DMSO) δ 166.97, 165.35, 165.02, 161.68, 155.66, 153.76, 145.57, 143.21, 143.12, 141.35, 141.28, 136.01, 133.04, 131.88, 131.41, 131.08, 130.76, 129.15, 128.84, 128.28, 125.91, 107.33, 107.24, 107.16, 107.07, 104.15, 104.07, 53.27, 53.23.

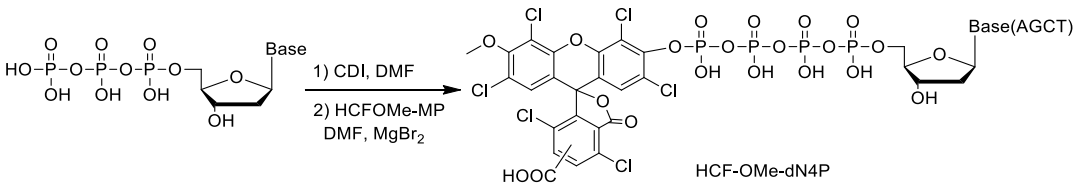
2) Preparation of **3′-O-Methyl-HCF (HCF-OMe)**



HCF-OMe was prepared by using the same procedure as 3′-O-Methyl-TFDCF.After hydrolysis the desire product was purified by silica gel chromatograph to give HCF-OMe as brown-red solid in 65% yield. ^{1}H NMR (500 MHz, DMSO) δ 8.26 (s, 1H-Ar for 5-isomer), 8.24 (s, 1H-Ar for 6-isomer), 7.29 (s, 2H-Ar for 6-isomer), 7.26 (s, 2H-Ar for 5-isomer), 3.53 (S, 6H-CH_3 for mixture); ^{13}C NMR (126 MHz, DMSO) δ 163.36, 163.28, 162.95, 162.72, 148.89, 141.64, 140.92, 135.25, 135.01, 134.14, 134.04, 132.18, 131.78, 130.77, 130.07, 129.80, 129.21, 128.26, 126.61, 125.98, 124.78, 124.71, 116.52, 112.59, 112.27, 108.85, 108.81, 51.88, 51.84. HRMS (ESI): M+1: 594.8468 (calc 594.8474 for $C_{22}H_9Cl_6O_7$)

III. Synthesis of Terminal Phosphate-Labeled Fluorogenic Nucleotides (TPLFNs)





1) Preparation of HCF-OMe-monophosphate

HCF-OMe (0.5g, 0.84 mmol) was suspended in 20 mL anhydrous CH_3CN under Ar(g). The mixture was cooled to -10 ^oC in salt-ice bath and phosphorous oxychloride (306 μL, 3.36 mmol) was added with stirring. After 10 min, Hünig's base (870 μL, 5.04 mmol) was added and the mixture was keeping stirred at -10 ^oC~0 ^oC for 2 h. Then TEAA buffer (1M, 15 mL) was added to quench the reaction and hydrolyze the formed phosphorous chloride intermediate. After 1 h stirring at 0 ^oC, the mixture was evaporated by using rotary evaporator to remove most of the CH_3CN, and the aqueous solution was filtered. The filtration was collected and HCF-OMe-monophosphate can be purified through revers phase C-18 flash column. The obtained elution containing the desired product was collected and concentrated in vacuo and co-evaporated twice with anhydrous DMF. The final pure product was re-dissolved in 4 mL anhydrous DMF to get the stock solution for further usage (~80 mM). MS (MALDI-TOF): M+1 = 674.50 (calc 674.81 for $C_{22}H_{10}Cl_6O_{10}P$).

2) Preparation of Terminal Phosphate-Labeled Fluorogenic Nucleotides (**HCF-OMe-dN4P**)

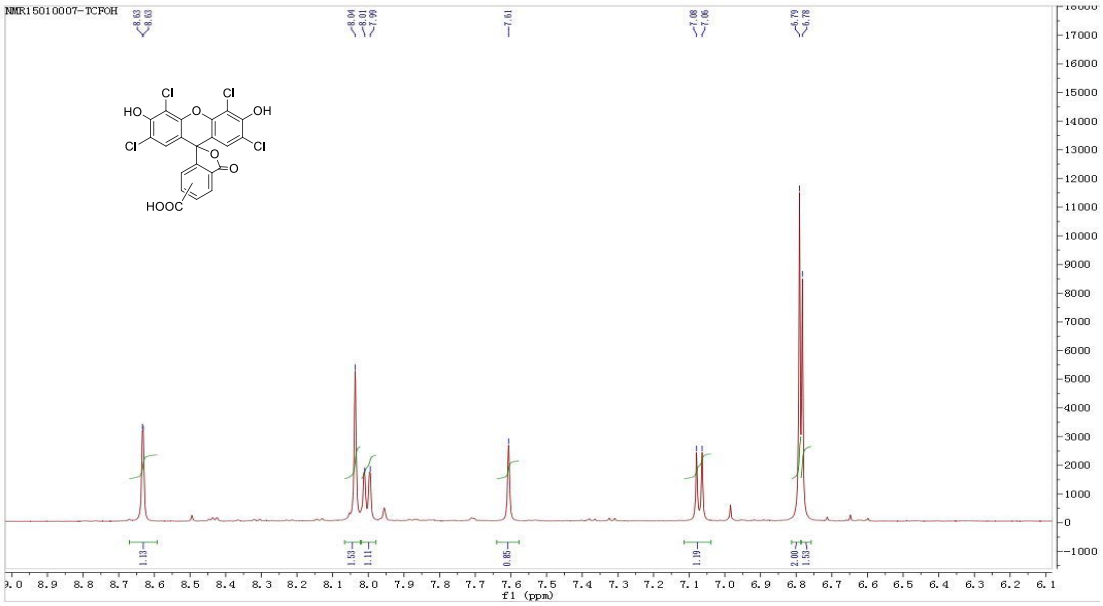
All the Phosphate-Labeled Fluorogenic Nucleotides were synthesized following the reported procedure[3] and purified on Shimadzu LC-20A HPLC system by using preparative reverse-phase sepax Amethyst C18-H column (21.2×150 mm) at 5 mL/min flow rate, with a gradient of B (CH_3CN) in A (50 mM TEAA pH 7.4) (0-15 % of B over 10 min, 15-35 % of B over 15 min, 35-50% of B over 10 min).All the correct products were verified by MALDI-TOF Mass Spectrometer.

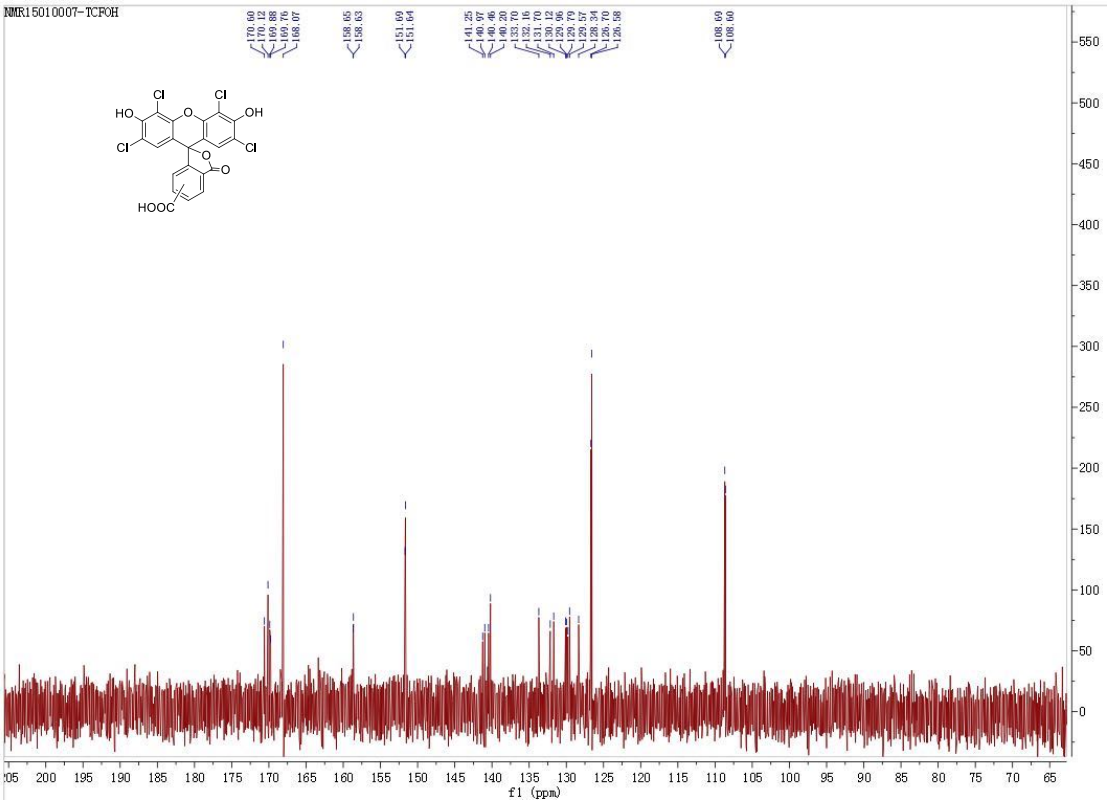
dA4P- δ -HCF-OMe: MS (MALDI-TOF): M-1 = 1147.85 (calc 1148.79)

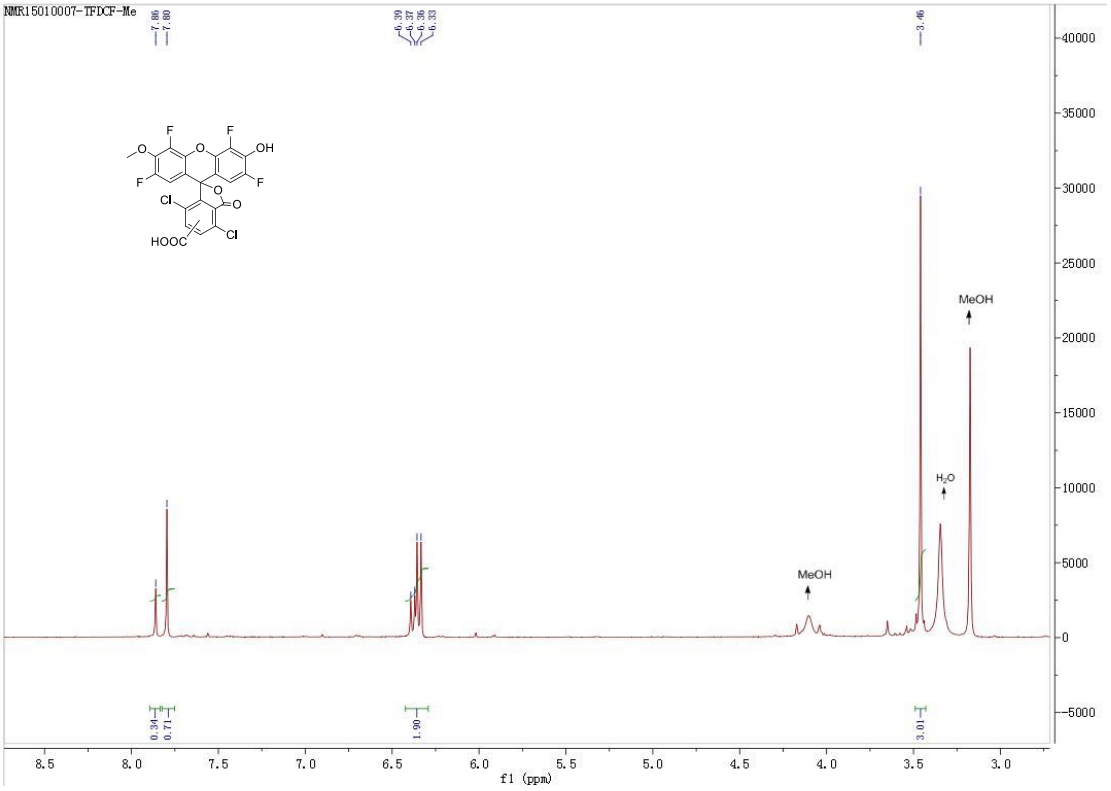
dC4P- δ -HCF-OMe: MS (MALDI-TOF): M-1 = 1124.01 (calc 1124.78)

dG4P- δ -HCF-OMe: MS (MALDI-TOF): M-1 = 1163.92 (calc 1164.79)

dT4P- δ -HCF-OMe: MS (MALDI-TOF): M-1 = 1124.83 (calc 1125.76)



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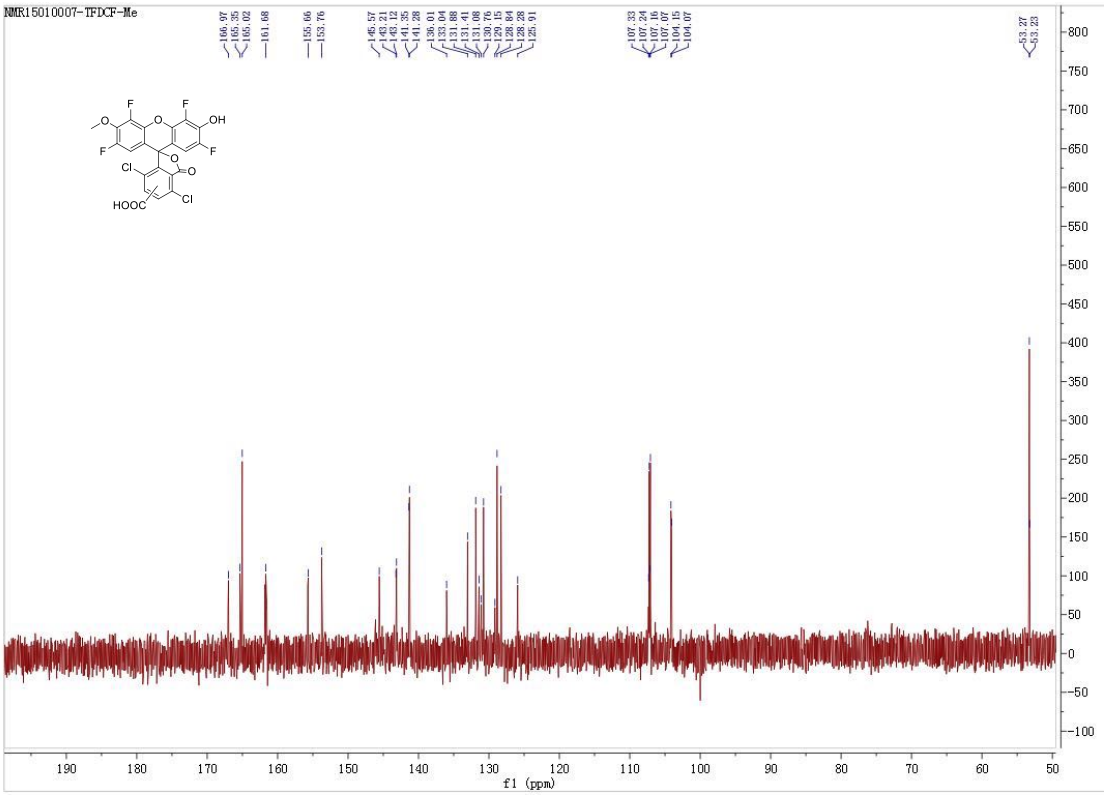
7.80
6.39
6.37
6.36
6.33
3.46
MeOH
H2O
MeOH
0.34
0.71
1.90
3.01
f1 (ppm)


NMR15010007-TFDCF-Me
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165.02
161.68
155.66
153.76
145.57
143.21
143.12
141.35
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136.01
133.04
133.00
131.41
131.08
130.76
129.15
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104.07
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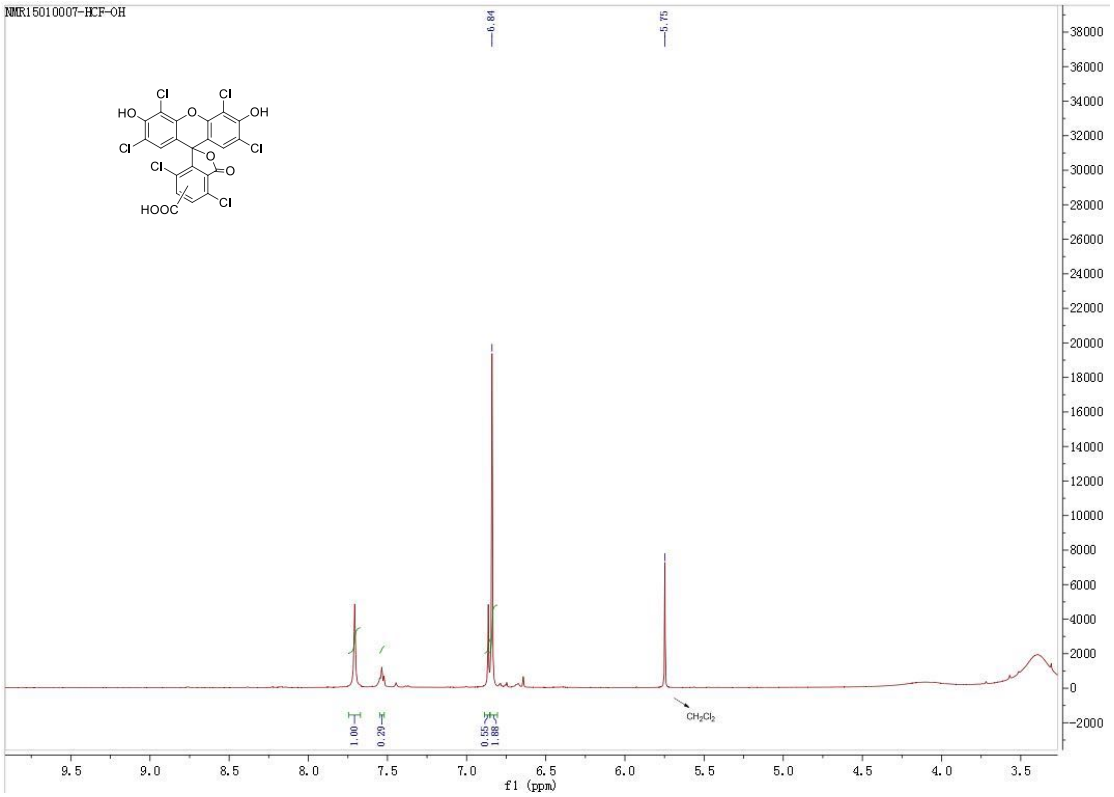
53.23
f1 (ppm)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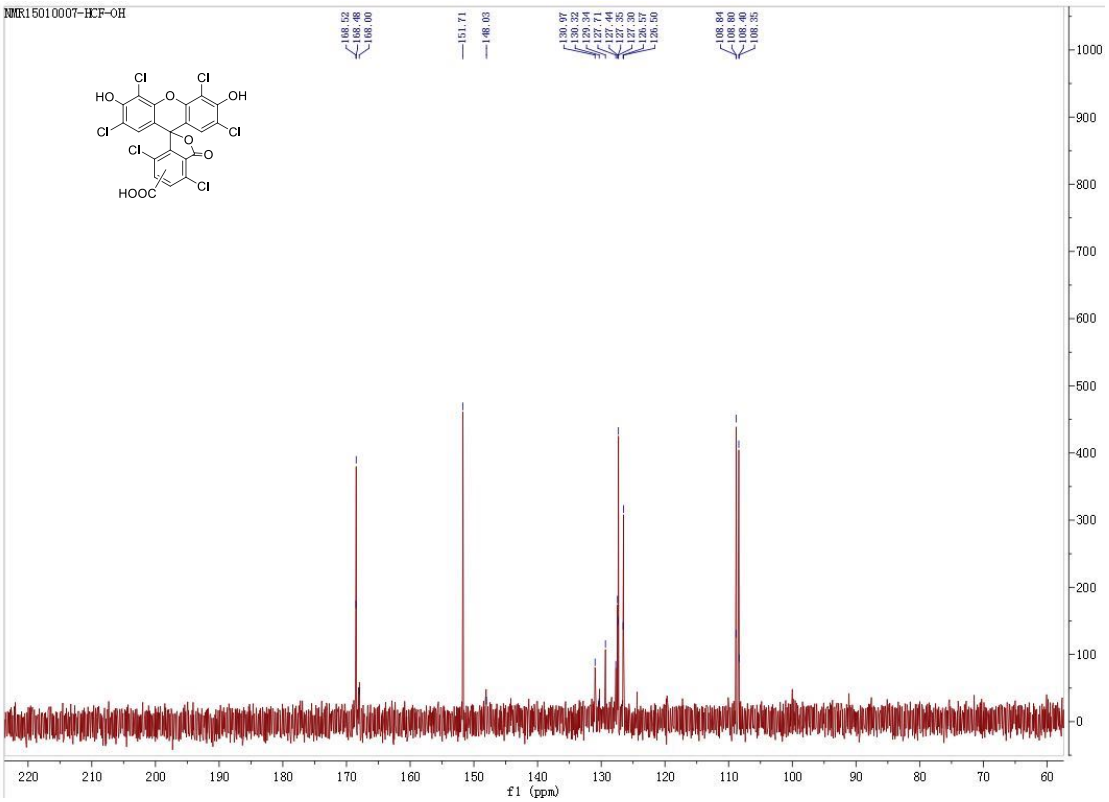
1.88
CH2Cl2
f1 (ppm)


NMR15010007-HCF-OH
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168.48
168.00
151.71
148.03
130.97
130.32
129.34
127.71
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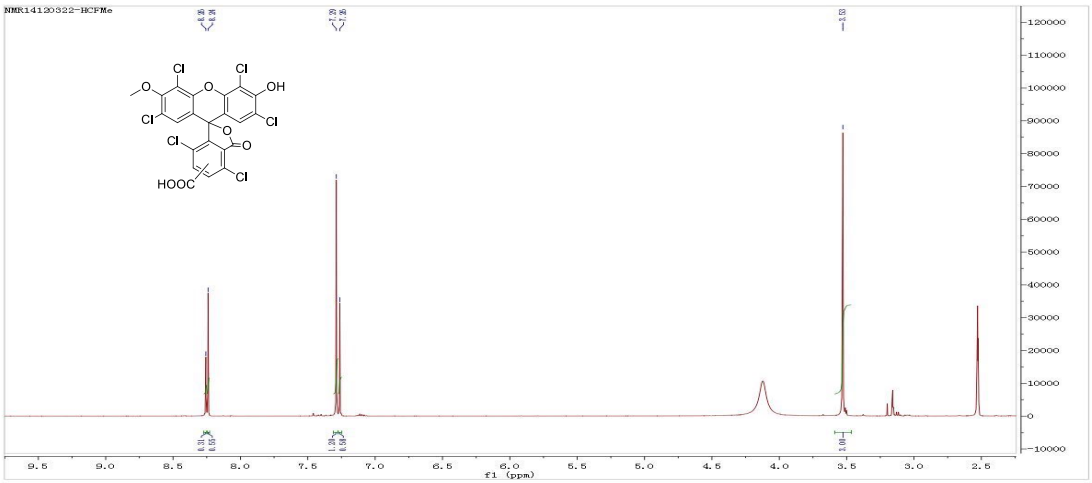
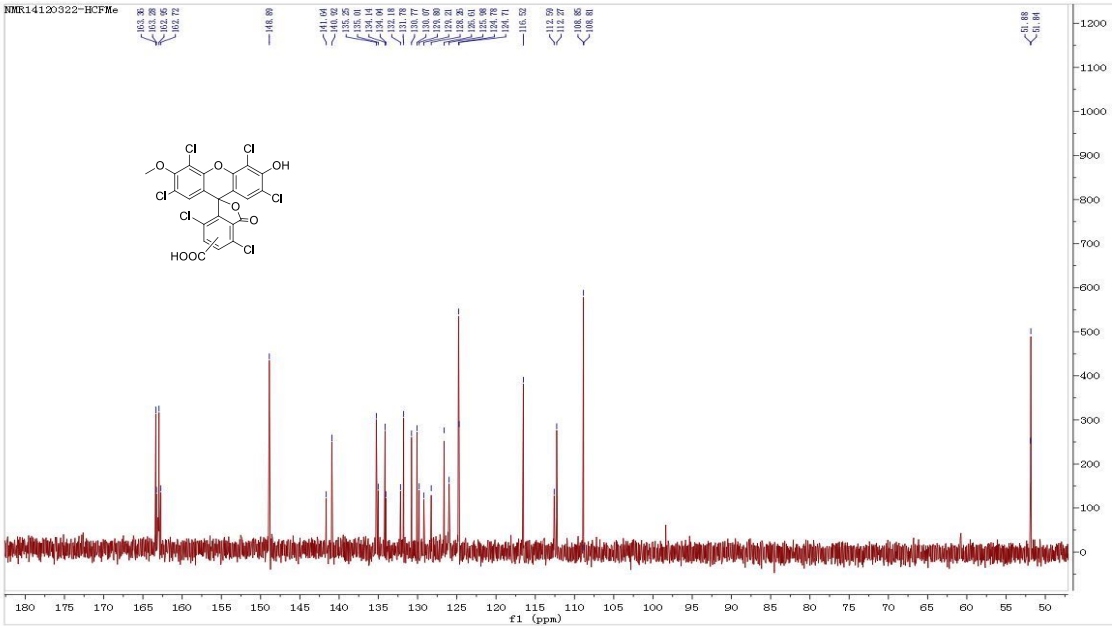
108.80
108.40
108.35
f1 (ppm)





Reference:

1. M. H. Lyttle, T. G. Carter, R. M. Cook, *Organic Process Research & Development* 2001, *5*, 45-49.
2. W.C. Sun, K.R. Gee, D.H. Klaubert, R.P. Haugland, *J. Org. Chem*. 1997, *62*, 6469–6475.
3. P. A Sims, W. J Greenleaf, H. Duan, X. Xie, *Nat Methods*. 2011, *8*, 575-580.

Section 2. Instrumentation for Measuring Kinetics

2.1 Home made kinetic measurement instrument

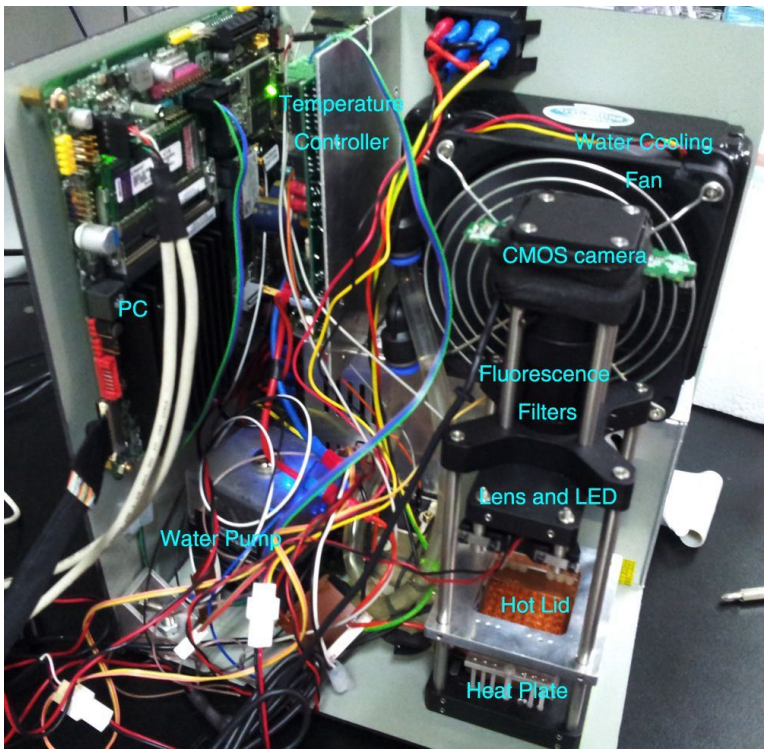


Fig S2.1. A home-developped kinetic measurement instrument. It contains a LED light source for excitation, a suitable filter which collect fluorescence efficiently, a CMOS camera for collecting fluorescence light, and a heat adaptor for hold and heat the plate. All componenets and pars are covered by a dark box during experiment.

Well intensity is calculated as the mean value of the circular area of each wells from fluorescence image. Calibration of each well was carried out using standard plate fill with 0 and 10 μM dye molecule in the same buffer used in kinetic experiment. A linear concentration-intensity function was then given by fitting with the two calibrated points. The accuracy of concentration assessment using this instrument was measured to be ~5%.

2.2 Method for combinatorial single-base extension measurement

The aim of this experiment was to measure the cross reaction of different nucleotide – base combination. Four oligonucleotides (sA1, sT1, sG1, and sC1, 20 μM in Tris buffer pH 8.0) were annealed with same volume of oligonucleotide (sP, 20 μM in Tris buffer pH 8.0) separately, forms four DNA 5′ over hangs (10 μM in Tris buffer pH 8.0).

Sequences are listed below.

Table S2.1. DNA sequence used in combinatorial one-base extension experiment

Name	Sequence
sP	CCTGTGTGCCTGCCTATCCCGTTGCGTGTCTCAG
sA1	CGTACTGAGACACGCAACGGGATAGGCAGGCACACAGG
sT1	CGATCTGAGACACGCAACGGGATAGGCAGGCACACAGG
sG1	AATGCTGAGACACGCAACGGGATAGGCAGGCACACAGG
sC1	TGACCTGAGACACGCAACGGGATAGGCAGGCACACAGG

Each reaction had a final volume of 20 μL. HCF-TPLFNs were diluted into 100 μM prior for use. Final recipe of enzyme master mix is shown below.

Table S2.2. Recipe of combinatorial one-base extension reaction mix

Component	Final Concentration
ThermoPol® Reaction Buffer(10 x)	1 X
$MnCl_2$	2 mM
Bst (NEB, M0275S, 8 kU/mL)	0.16 U/μL
CIP (NEB, M0290S, 10 kU/mL)	0.2 U/μL
DNA overhangs (20 μM)	400 nM
HCF-TPLFNS (100 μM)	2 μM

We made 2 plates for providing technical replicates for each reaction.

2.3 Method for misincorporation test.

M13mp18ss DNA was purchased from NEB (N4040S). Four reversed primers used here were designed using PrimerBlast tool.

Table S2.3. primer sequence

Name	Sequence
M13mp18-6614	ACAACCCGTCGGATTCTCCGTGGGA
M13mp18-5411	GAGCGGGAGCTAAACAGGAGGCCGA
M13mp18-3332	GCGAGGCGTTTTAGCGAACCTCCCG
M13mp18-992	AGGCGCATAGGCTGGCTGACCTTCA

Four primers were annealed with M13mp18 ssDNA in advance, making four different long-extendable DNA substrates and diluted into 100 nM. Master mix contained all four HCF-TPLFNs.

Table S2.4. Recipe of misincorporation test reaction mix

Component	Final Concentration
ThermoPol® Reaction Buffer(10x)	2 X
$MnCl_2$	2 mM
Bst (NEB, M0275S 8kU/mL)	0.24 U/μL
CIP (NEB, M0290S 10kU/mL)	0.1 U/μL
ssDNA w/ primer (100nM)	0.5 nM
HCF-TPLFNS (100μM)	5 μM each

We made 8 replicates for each primer, in 4 rows, and 8 negative control replicates using ssDNA (without the annealed primer).

Section 3. Sequencing.

We used asymmetry solid phase PCR to generate surface immobilized DNA templates. We injected PCR mix (Table S3.1) into the flow chamber after grafting with solid phase primer PS1. We then performed 15~20 cycles of pre-amplification (95 °C 30 s, 65 °C 15 s, 72 °C 45 s) to generate enough single strand DNA templates and another 20~30 cycles of amplification (95 °C 30 s, 65 °C, 150 s) to anneal and extend pre-amplified ssDNA onto surface primer efficiently. After PCR, we washed the chip using DI water for 10 min. Typically at least 2 fmol/mm^2 of template would be amplified onto surface.

Table S3.1 recipe of solid-phase PCR mix

Component	Concentration
Primer PW1	1 μM
Primer PW2	125 nM
DNA template	100 pM
Platinum Taq (Life technology)	0.3 U/μL
Mg^{2+}	3 mM
Standard Taq Buffer	1 x

Table S3.2 DNA sequence used in solid-phase PCR (5′ to 3′)

Name	Sequence
PS1	(AMN) TTTTTTTTTTCAAGCAGAAGACGGCATACGAGAT
PW1	AATGATACGGCGACCAC
PW2	CAAGCAGAAGACGGCATA
template	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCTGTGTTCGACGGTGAGCTGAGTTTTGCCCTGAAA CTGGCGCGTGAGATGGGGCGACCCGACTGGCGTGCCATGCTTGCC GGGATGTCATCCACGGAGTATGCCGACTGGCACCGCTTTTACAGT ACCCATTATTTTCATGATGTTCTGCTGGATATGCACTTTTCCGGGC TGACGTACACCGTGCTCAGCCTGTTTTTCAGCAAGATCGGAAGAG CACACGTCTGAACTCCAGTCACGTCAGTATCTCGTATGCCGTCTTC TGCTTG

We simply prepared four nucleotides mixes and one wash buffer as washing reagent. Each cycle of sequencing reaction will consume ca. 50 μL reaction mix and 5 mL wash buffer. Recipe of wash buffer is similar as 1X isothermal Amplification buffer (US, NEB, B0537S), with 1 mM EDTA but without $MgSO_4$. Reaction mix contains TPLFN, CIP and *Bst* polymerase in 1X isothermal Amplification buffer. Instead of Mg^{2+} ion, we here used Mn^{2+} as cofactor for polymerase.

Degradation of raw signals were firstly corrected using method shown in our previous work, followed by a dephasing correction step using ‘CAFIE algorism’ (Patent US 8301394).