Terminal Phosphate-Labeled Nucleotides with Improved Substrate Properties for Homogeneous Nucleic Acid Assays

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Terminal Phosphate-Labeled Nucleotides with Improved Substrate Properties for Homogeneous Nucleic Acid Assays

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It is now common practice to use nucleotides labeled in either the base or sugar regions as precursors for the synthesis of labeled RNA and DNA. In fact, base-labeled nucleoside-5′-triphosphates are key components of many commercial nucleic acid assay kits that are routinely used for DNA sequencing, gene expression analysis, and genotyping.[1] For example, dideoxynucleotides fluorescently labeled with energy-transfer dyes via a propargylamino linker at the C-5 position of pyrimidines and C-7 position of 7-deazapurines are now the most commonly used chain terminators for DNA sequencing.[2] Incorporation of these fluorescent nucleotides by DNA polymerases terminates and labels the newly synthesized DNA chain.

Measuring of the other product of DNA or RNA synthesis, namely the pyrophosphate, has been largely ignored, being useful only for detecting the presence or absence of synthesis in such techniques as pyrosequencing.[3] However, recent interest in single-molecule sequencing has renewed interest in detecting the identity of a newly added base without modification of the product DNA structure.[4] We have found a simple and elegant way to determine the identity and quantity of nucleotides added to a growing chain using the following elements:

1. Polyphosphate chains esterified at both ends are inert to hydrolysis catalyzed by alkaline phosphatase, while chains with esters at only one end are rapidly hydrolyzed.

2. There are many dyes which change color or fluorescence when converted from an alcohol (−OH) form to a phosphate ester.

3. While nucleoside triphosphates labeled on the terminal phosphate are relatively poor substrates for RNA and DNA polymerases, the analogous tetraphosphates and pentaphosphates have 1−2 orders of magnitude better activity, depending on the specific polymerase and nucleotide (Figure 2).

Together, these elements allow us to devise assays for a wide range of applications including sequence detection and genotyping with sensitive, convenient, homogeneous assay formats.

These assays use a new class of phosphate-labeled nucleotides (Figure 1) which not only possess the desirable properties of phosphate-labeled nucleotides but also are incorporated at least an order of magnitude faster by DNA polymerases than γ-modified nucleoside triphosphates. These nucleotides possess more than three phosphates, and the terminal phosphate is labeled with a dye or other moiety through an OH group.

The synthesis of terminal phosphate-labeled nucleotides involves the use of a nucleoside-5′-triphosphate and a labeling dye having either a free −OH group or a phosphate ester.[5] For the synthesis of γ-labeled triphosphates, the required nucleoside-5′-triphosphate is reacted with dicyclohexylcarbodiimide (DCC) to give the cyclic triphosphate, which is then reacted with the dye having a free OH group. The synthesis of tetra- (δ) or penta- (ϵ) phosphate-labeled nucleotides is carried out by reacting activated dye−monophosphate or dye−pyrophosphate with nucleoside-5′-triphosphate in 50−75% yield. For many applications of these phosphate-tagged nucleotides, a reaction mixture is prepared containing one or more tagged nucleotides, a polymerase, alkaline phosphatase, and an appropriate nucleic acid template. At the start of the reaction, the tagged nucleotides are inert to hydrolysis by alkaline phosphatase and are nonfluorescent (or emit very weakly or at different wavelengths). Once the nucleotide is incorporated into DNA or RNA by polymerase, a polyphosphate dye moiety is released. This is rapidly hydrolyzed by alkaline phosphatase to release the −OH form of the dye, which is strongly fluorescent or otherwise readily detectable. The spectral properties of some of the dyes we have attached

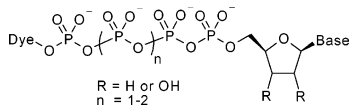


Figure 1. General structure of terminal phosphate-labeled nucleotides.

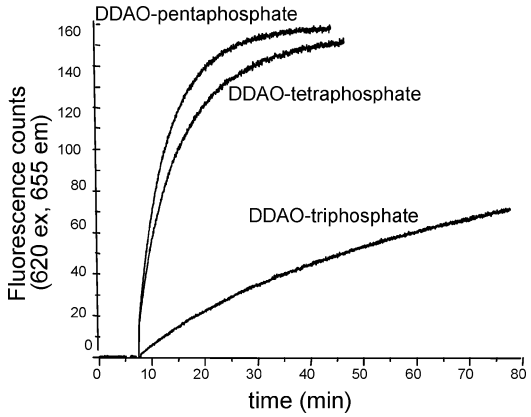
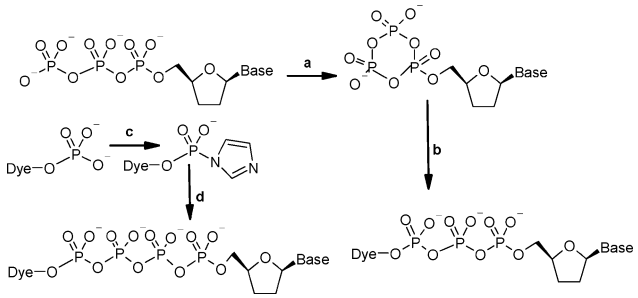


Figure 2. Depending on the DNA polymerase, nucleotide base, and reaction conditions, labeled tetra- and pentaphosphates give rates that are 10−50 times faster than those obtained with labeled triphosphates. Assays using *Taq* DNA polymerase at 42° with normal triphosphates give a rate of nucleotide addition of 1.5 s^{-1}. With unlabeled dT4P the rate is about 0.25 s^{-1}, and that with labeled dT4P is about 0.22 s^{-1}.

Scheme 1. Synthesis of Terminal Phosphate-Labeled Nucleotides[a]



[a] (a) DCC, DMF. (b) Dye-OH. (c) CDI, DMF. (d) Nucleoside triphosphate.

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Table 1. Fluorescence Excitation and Emission Wavelengths (nm) of Dye−Nucleotide Conjugates and the Free Dyes Released by Enzymatic Reaction

	labeled nucleotide		free dye anion	
dye	excitation	emission	excitation	emission
4-Me-coumarin	319	383	360	449
3-cyanocoumarin	356	411	408	450
ethyl fluorescein	274	nonfl	456	517
Resorufin	473	nonfl	570	580
DDAO	455	615	645	660

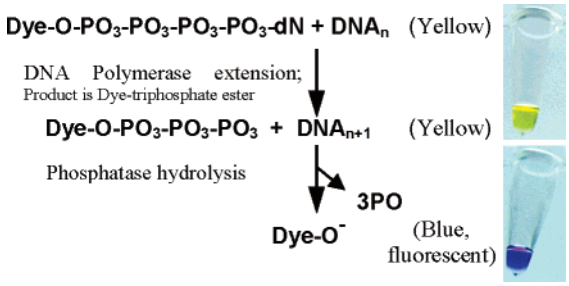

Figure 3. Combination of polymerase and phosphatase can result in homogeneous assays using suitable dyes. Suitable dyes are ones that change spectral characteristics between ester and anion forms.

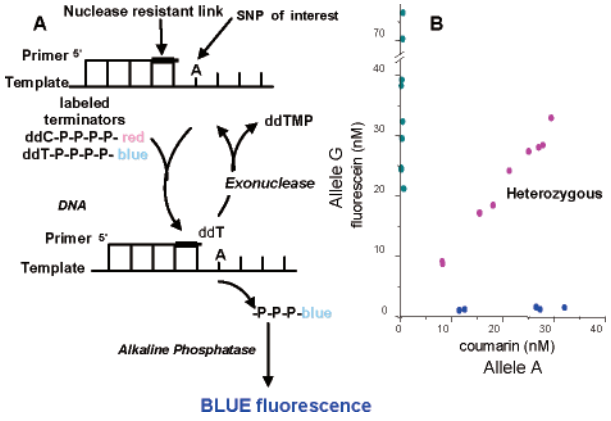

Figure 4. (A) An amplifying, homogeneous assay scheme for SNP typing using terminal phosphate-labeled nucleotides. Assays can contain two or four different dye-labeled nucleotides. (B) Assays for human SNP TSC0000431 using DNA from 12 different individuals.

to the terminal phosphate, which only become fluorescent when released, are given in Table 1.

The change in spectral property can be very easily detected by a variety of fluorescence instruments such as plate-readers and scanners. It can even be visualized by the naked eye when a high concentration of DNA or amplification is used. For example, 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) attached to terminal phosphate of dTTP has yellow color. When DNA polymerase incorporates the nucleotide during rolling circle amplification (RCA),[6] the released pyrophosphate (still yellow) changes to blue only after hydrolysis with phosphatase (Figure 3). Thus, these color-changing, terminal phosphate-labeled nucleotides can be exploited in homogeneous assays for polymerases or for specific templates.

An example of such an assay is shown in Figure 4. This is an assay for a specific single nucleotide polymorphism (SNP) performed using phosphate-tagged dideoxynucleotides. First, polymerase chain reaction (PCR) is used to amplify the region containing the SNP from an individual. This PCR product DNA (a linear, double-stranded DNA, approximately 0.1−0.5 Kb) is then mixed with a primer whose 3′ end is adjacent to the SNP, along with at least two dye-tagged nucleotides, one for each of the expected alleles. After annealing the primer, a DNA polymerase can extend it using one or both of the nucleotides, depending on the bases present in the template strand. One color will indicate one homozygous allele; both colors indicate a heterozygous result. The signal can be further amplified by using a 3′ exonuclease such as *Escherichia coli* exonuclease III to remove the added dideoxynucleotide so that polymerase can add it repeatedly, generating more fluorescent dye. For this to be practical, a primer that is resistant to nuclease digestion, such as one containing phosphorothioates, is substituted as shown. Using *E. coli* exonuclease III, we have achieved more than 1000-fold amplification in 1 h. In over 80 independent human SNP assays, correct results were obtained 100% of the time, even when multiplex PCR was used for the initial step.

In addition to the SNP genotyping assays described, a wide variety of other applications are possible using these nucleotides. For example, we have been able to sequence templates using repeated addition of single labeled nucleotides to a primer template attached to a solid surface.[7] We have also been able to quantify both specific and nonspecific amplification of DNA using PCR and RCA. In this way, quantitative PCR with enhanced sensitivity is possible.[8] In addition, polymerase template oligonucleotides could be attached to virtually any kind of analyte or binding protein for use in immunoassays or array assays. We expect that this new approach to assay design will find applications in many important fields.

Supporting Information Available: Details of synthesis and characterization of labeled nucleoside polyphosphates, enzymatic incorporation, and assay conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Lee, L. G.; Connell, C. R.; Woo, S. L.; Cheng, R. D.; McArdle, B. F.; Fuller, C. W.; Halloran, N. D.; Wilson, R. K. *Nucleic Acids Res.* **1992**, *20*, 2471−2483. (b) Duthie, R. S.; Kalve, I. M.; Samols, S. B.; Hamilton, S.; Livshin, I.; Khot, M.; Kumar, S.; Fuller, C. W. *Bioconjugate Chem.* **2002**, *13*, 699−706. (c) Kumar, S. In *Modified Nucleotides, synthesis and applications*; Loakes, D., Ed.; Research Signpost: Trivandrum (India), 2002; pp 87−100 and references therein.

(2) (a) Nampalli, S.; Khot, M.; Kumar, S. *Tetrahedron Lett.* **2000**, *41*, 8867−8871. (b) Rosenblum, B. B.; Lee, L. G.; Spurgeon, S. L.; Khan, S. H.; Menchen, S. M.; Heiner, C. R.; Chen, S. M. *Nucleic Acids Res.* **1997**, *25*, 4500−4504.

(3) (a) Ronaghi, M.; Uhlen, M.; Nyren, P. *Science* **1998**, *281*, 363−365. (b) Ronaghi, M.; Nygren, M.; Lundeberg, J.; Nyren, P. *Anal. Biochem.* **1999**, *267*, 65−71. (c) Ronaghi, M. *Genome Res.* **2001**, *11*, 3−11.

(4) Levene, M. J.; Korlach, J.; Turner, S. W.; Foquet, M.; Craighead, H. G.; Webb, W. W. *Science* **2003**, *299*, 682−686.

(5) (a) Arzumanov, A. A.; Semizarov, D. G.; Victorova, L. S.; Dyatkina, N. B.; Krayevsky, A. A. *J. Biol. Chem.* **1996**, *271* (40), 24389−24394. (b) Knorre, D. G.; Kurbatov, V. A.; Samukov, V. V. *FEBS Lett.* **1976**, *70* (1), 105−108.

(6) (a) Detter, J. C.; Jett, J. M.; Lucas, S. M.; Dalin, E.; Arellano, A. R.; Wang, M.; Nelson, J.; Chapman, J.; Lou, Y.; Rokhsar, D.; Hawkins, T. L.; Richardson, P. M. *Genomics* **2002**, *80* (6), 691−698. (b) Richardson, J. C.; Detter, C.; Schweitzer, G.; Predki, P. F. *Genet. Eng. (N.Y.)* **2003**, *25*, 51−63.

(7) (a) Sood, A.; Kumar, S.; Nelson, J. R.; Fuller, C. W. Patent PCT WO 020734, 2003. (b) Sood, A.; Kumar, S.; Nelson, J. R.; Fuller, C. W. Patent PCT WO 020604, 2004.

(8) Sood, A.; Kumar, S.; Nelson, J. R.; Fuller, C. W.; Sekher A. Patent PCT WO 072304, 2004.

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SUPPLEMENTARY MATERIAL

Terminal Phosphate Labeled Nucleotides with Improved Substrate Properties for Homogenous Nucleic Acid Assays

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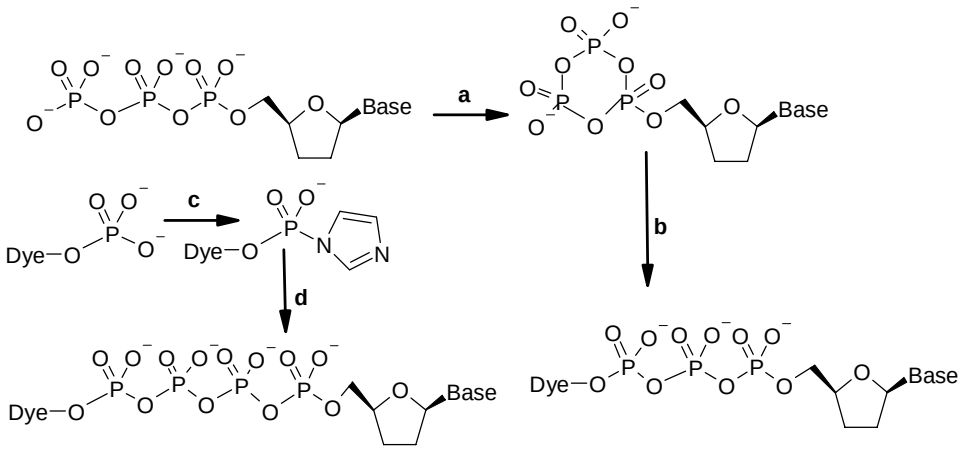
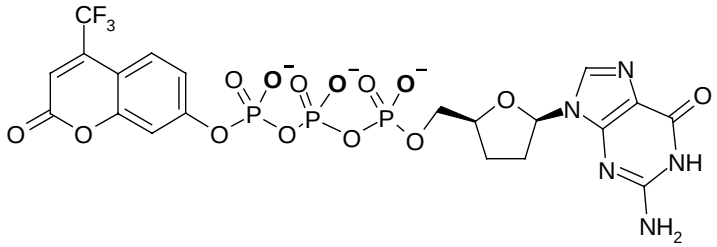
1) Synthesis of Terminal phosphate Labeled Nucleotides



a. DCC, DMF. b. Dye-OH. c. CDI, DMF d. nucleoside triphosphate

(a) Synthesis of γ-(4-trifluoromethylcoumarinyl)ddGTP (γ-CF_3Coumarin-ddGTP)

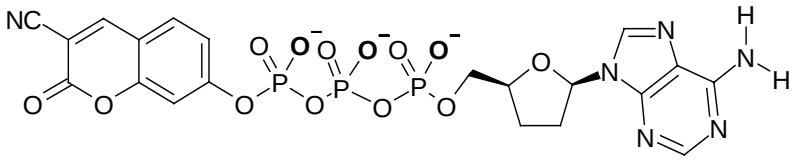


γ-(4-trifluoromethylcoumarinyl)dideoxyguanosine-5'-triphosphate
(γCF_3Coumarin-ddGTP)

ddGTP (200 µl of 46.4 mM solution, purity >96%) was coevaporated with anhydrous dimethylformamide (DMF, 2x 0.5 ml). To this dicyclohexylcarbodiimide (DCC, 9.6 mg, 5 eq.) was added and mixture was again coevaporated with anhyd. DMF (0.5 ml). Residue was taken in anhyd. DMF (0.5 ml) and mixture was allowed to stir overnight. To the mixture another 2 eq. of DCC was added and after stirring for 2h, 7-hydroxy-4-trifluoromethyl coumarin (4-trifluoromethyl-umbelliferone, 42.7 mg, 20 eq.) and triethylamine (26 µl, 20 eq.) were added and mixture was stirred at RT. After 2 days, HPLC (0-30% acetonitrile in 0.1M triethylammonium acetate (TEAA)

in 15 minutes, 30-50 % acetonitrile in 5 min and 50-100% acetonitrile in 10 minutes, C18 3.9x150 mm column, flow rate 1 ml/minute) showed a new product at 9.7 min and starting cyclic triphosphate (ratio of 77 to 5 at 254 nm). Mixture was allowed to stir for another day. P-31 NMR showed gamma labeled nucleoside-triphosphate as the main component of reaction mixture. Reaction mixture was concentrated on rotary evaporator. Residue was extracted with water (5x1 ml) and purified on 1 inch x 300 cm C18 column using 0-30% acetonitrile in 0.1M triethylammonium bicarbonate (TEAB, pH 8.3) in 30 min and 30-50% acetonitrile in 10 min, 15 ml/min flow rate. Product peak was concentrated and coevaporated with MeOH (2 times) and water (1 time). Sample was dissolved in 1 ml water. HPLC showed a purity of > 99% at 254 and 335 nm. UV showed a conc. of 2.2 mM assuming an extinction coefficient of 11,000 at 322 nm. MS: M- = 702.18 (calc 702.31), UV λ max= 253, 276 & 322 nm.

(b) Synthesis of γ - (3-Cyanocoumarinyl)ddATP (γ-CNCoumarin-ddATP)



γ–(3-cyanocoumarinyl)dideoxyadenosine-5'-triphosphate
(γCNCoumarin-ddATP)

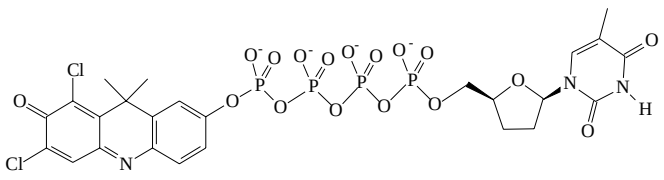
ddATP (100 µl of 89 mM solution, >96%) was coevaporated with anhydrous DMF (2x 1 ml). To this DCC (9.2 mg, 5 eq.) was added and mixture was again coevaporated with anhydrous DMF (1 ml). Residue was taken in anhydrous DMF (0.5 ml) and reaction was stirred at rt. After overnight, 7-hydroxy-3-cyanocoumarin (33.3 mg, 20 eq.) and TEA (25 µl, 20 eq.) were added and mixture was stirred at room temperature . After 1 day, a major product (55% at 254 nm) was observed at 8.1 min with another minor product at 10 min (~10%). No significant change occurred after another day. Reaction mixture was concentrated on rotary evaporator and residue was extracted with water (3x2 ml), filtered, concentrated and purified on C-18 using a gradient of 0-30% acetonitrile in 0.1M TEAB (pH 6.7) in 30 min and 30-50% acetonitrile in 10 min, flow rate 15 ml/min. Main peak was collected in 3 fractions. HPLC of the main peak (fr. 2) showed a purity of 95.6% at 254 nm and 98.1% at 335 nm. It was concentrated on rotary evaporator, coevaporated with MeOH (2x) and water (1x). Residue was dissolved in 0.5 ml water. Assuming an extinction coefficient of 20,000 (reported for 7-ethoxy-3-cyanocoumarin, Molecular Probes Catalog), concentration = 7.84 mM. Yield = 3.92 µmol, 44%. Sample was repurified on C-18 column using same method as above and pure fractions were combined. After concentration,

residue was coevaporated with MeOH (2x) and water (1x). Sample was dissolved in water (1 ml) to give a 2.77 mM solution. MS: M- = 642.98 (calc 643.00), UV λmax = 263 & 346 nm

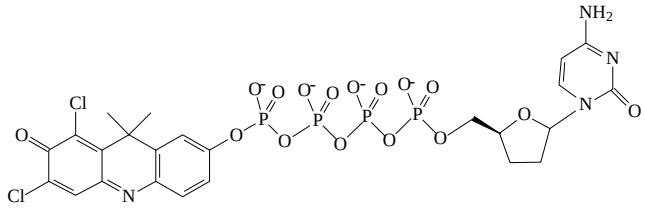
(c) Synthesis of δ-9H(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)-dideoxythymidine-5'-tetraphosphate (ddT4P-DDAO)

ddTTP (100 µl of 80 mM solution) was coevaporated with anhydrous dimethylformamide (DMF, 2x 1 ml). To this dicyclohexylcarbodimide (8.3 mg. 5 eq.) was added and the mixture was again coevaporated with anhydrous DMF (1 ml). Residue was taken in anhydrous DMF (1 ml) and reaction was stirred at room temperature overnight. HPLC showed mostly cyclized triphosphate (~82%). Reaction mixture was concentrated and residue was washed with anhydrous diethyl ether (3 x). It was redissolved in anhydrous DMF and concentrated to dryness on rotavap. Residue was taken with DDAO-monophosphate, ammonium salt (5 mg, 1.5 eq.) in 200 µl anhydrous DMF and stirred at 40ºC over the weekend. HPLC showed formation of a new product with desired UV characteristics at 11.96 min. (HPLC Method: 0.30% acetonitrile in 0.1M triethylammonium acetate (pH 7) in 15 min, and 30-50% acetonitrile in 5 min, Novapak C-18 3.9x150 mm column, 1 ml/min). LCMS (ES-) also showed a major mass peak 834 for M-1 peak. Reaction mixture was concentrated and purified on Deltapak C18, 19x 300 mm column using 0.1M TEAB (pH 6.7) and acetonitrile. Fractions containing product were repurified by HPLC using the same method as described above. The appropriate fractions were concentrated, coevaporated with MeOH (2x) and water (1x). Residue was dissolved in water (1.2 ml) to give a 1.23 mM solution. HPLC purity at 254 nm > 97.5%, at 455 nm > 96%; UV λmax = 267 nm and 455 nm; MS: M-1 = 833.85 (calc 833.95).

δ-9H(1,3-dichloro-9,9-dimethylacridin-2-one-7=yl)-dideoxycytidine-5'-tetraphosphate (ddC4P-DDAO), δ-9H(1,3-dichloro-9,9-dimethylacridin-2-one-dideoxyadenosine-5'-tetraphosphate (ddA4P-DDAO) and δ-9H(1,3-dichloro-9,9-dimethylacridin-2-one-y-YL)-dideoxyguanosine-5'-tetraphosphate (ddG4P-DDAO) were synthesized and purified in a similar fashion. Analysis of these purified compounds provided the following data: ddC4P-DDAO: UV λmax = 268 nm and 454 nm; MS: M-1 = 819.32 (calc 818.96); ddA4P-DDAO: UV λmax = 263 nm and 457 nm; MS: M-1 = 843.30 (calc 842.97); ddG4P-DDAO: UV λmax = 257 nm and 457 nm; MS: M-1 = 859.40 (calc 858.97).



ddT4P-DDAO



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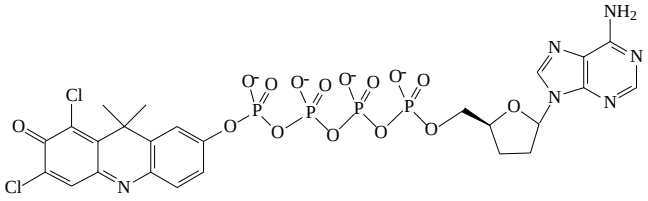

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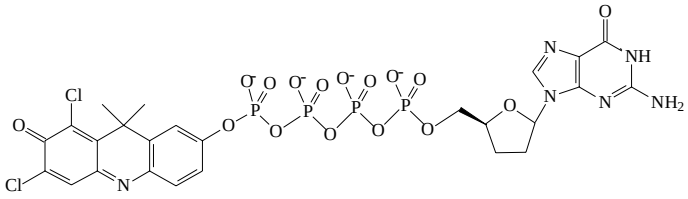
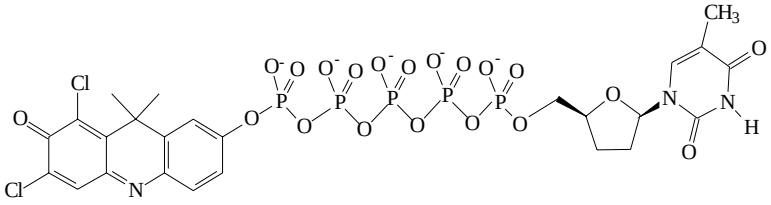


ddG4P-DDAO

Synthesis of ε-9H (1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)-dideoxythymidine-5'-pentaphosphate; DDAO-ddT-pentaphosphate (ddT5P-DDAO)



ddT5P-DDAO

a. Preparation of DDAO pyrophosphate

DDAO-phosphate diammonium salt (11.8 µmol) was coevaporated with anhydrous DMF (3x 0.25 ml) and was dissolved in DMF (0.5 ml). To this carbonyldiimidazole (CDI, 9.6 mg, 5 eq) was added and the mixture was stirred at room temperature overnight. Excess CDI was destroyed by addition of MeOH (5 µl) and stirring for 30 minutes. To the mixture tributylammoniumdihydrogen phosphate (10 eq., 236 ml of 0.5 M solution in DMF) was added and the mixture was stirred at room temperature for 4 days. Reaction mixture was concentrated on rotavap. Residue was purified on HiPrep 16.10 Q XL column using 0-100% B using 0.1M

TEAB/acetonitrle (3:1) as buffer A and 1 M TEAB/acetonitrile (3:1) as buffer B. Main peak (HPLC purity 98%) was collected, concentrated and coevaporated with methanol (2x). Residue was dissolved in 1 ml water to give 5.9 mM solution. UV/VIS λmax = 456 nm.

b. Preparation of ddT5P-DDAO

ddTTP (100 µl of 47.5 mM solution in water) was coevaporated with anhydrous DMF (2x1 ml). To this DCC (5 eq., 4.9 mg) was added and mixture was coevaporated with DMF (1x1 ml). Residue was taken in anhydrous DMF (0.5 ml) and stirred at room temperature for 3 hours. To this 1.03 eq of DDAO pyrophosphate, separately coevaporated with anhydrous DMF (2x1 ml) was added as a DMF solution. Mixture was concentrated to dryness and then taken in 200 µl anhydrous DMF. Mixture was heated at 38ºC for 2 days. Reaction mixture was concentrated, diluted with water, filtered and purified on HiTrap 5 ml ion exchange column using 0-100% A-B using a two step gradient. Solvent A = 0.1M TEAB/acetonitrile (3:1) and solvent B = 1M TEAB/acetonitrile (3:1). Fractions containing majority of the product were combined, concentrated and coevaporated with methanol (2x). Residue was repurified on Xterra RP C-18 30-100 mm column using 0.30% acetonitrile in 0.1M TEAB in 5 column and 30-50% acetonitrile in 2 column volumes, flow rate 10 ml/min. Fraction containing pure product was concentrated and coevaporated with methanol (2x) and water (1x). HPLC purity at 455 nm > 99%. UV/VIS λmax = 268 nm and 455 nm. MS: M-1 = 913.98 (calc 913.93).

Extended Synthesis using all four terminal phosphate labeled dNTP's with or without linker between the dye and phosphates.

Using the same primer/template (shown above), two different types of modified nucleotides (optimized $(CH_2)_7$ linker or directly attached dye to the triphosphate) were tested for the extension reaction and formation of full length product. Reaction mixture contained 25 mM Tris, pH 8.0, 50 mM KCl, 0.7 mM MnCl2, 0.5 mM beta-mercaptoethanol, 0.1 mg/ml BSA, 0.005 unit/ul SAP, 75 nM each primer and template, 1 uM each DDAO-dNTP (where N = A, T, G, C) or four different rhodamine dye attached dNTPs with $(CH_2)_7$ linker, and 6 ul of 0.12 mg/ml Phi 29 exo- polymerase to start the reaction. Although the rate of synthesis is faster with Dye-$(CH_2)_7$-PPP-dN type of nucleotides, nucleic acid synthesis proceeds to completion with both types of modified nucleotides (Figure 1).

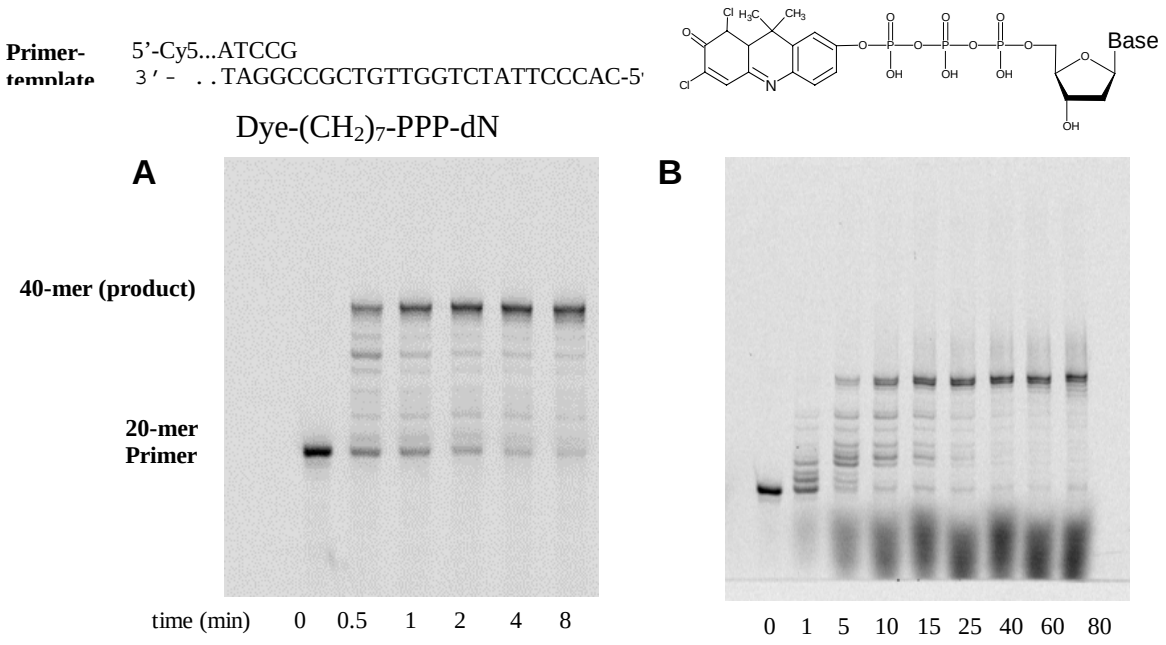


Figure 1: DNA synthesis using either linker based or directly attached dye to the terminal phosphate. The rate of synthesis is faster with Dye-$(CH_2)_7$-PPP-dN nucleotides.

Phosphate labeled nucleotides with more than 3 phosphates are better substrate for DNA polymerases.

Nucleoside-5'-triphosphates labeled on the terminal (γ) phosphate are relatively poor substrates for DNA and RNA polymerases. To assess the effect of extra phosphates on incorporation by DNA polymerases, we have synthesized and tested nucleotides with 4 or 5 phosphates. The rationale behind adding extra phosphate was to a) move dye away from the enzyme binding site, and/or b) to compensate for the loss of charge on phosphates upon attachment of dye to the triphosphate. The synthesis of these nucleotides was carried out as shown in Scheme 1 above. These tetra- and penta-phosphate labeled nucleotides are 1-2 orders of magnitude better substrates than the corresponding triphosphate as shown in Figure 2.

To assess the effect of extra phosphates, reactions containing 50 mM Tris, pH 8.0, 5% glycerol, 5 mM $MgCl_2$, 0.01% tween-20, 0.21 units shrimp alkaline phosphatase (SAP),100 nM primer annealed to template, and 1 μM ddT(P)n-DDAO were assembled in a quartz fluorescence ultra-microcuvet in a LS-55 Luminescence Spectrometer (Perkin Elmer), operated in time drive mode. Excitation and emission wavelengths are 620 nm and 655 nm respectively. Slit widths

were 5 nm for excitation slits, 15 nm for emission slits. The reaction was initiated by the addition of 0.35 µl (11 units) of a cloned DNA polymerase (Thy B) and 0.25 mM $MnCl_2$. As shown in Figure 4 (A), both tetra- and penta-phosphates are significantly better substrates than triphosphates. Figure 4 (B) shows the comparison of all four DDAO-labeled triphosphates with all four DDAO-labeled tetraphosphates. Again, tetraphosphates are upto 50 fold better substrates (depending on the nucleotide and polymerase used) than the corresponding triphosphates.

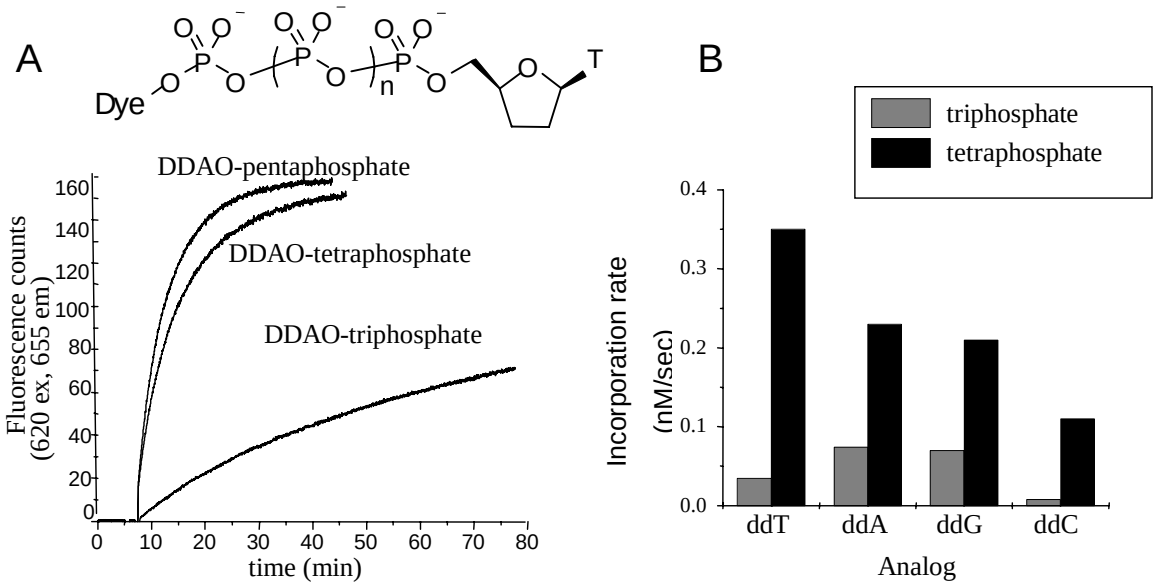


Figure 2: A) Effect of extra phosphate on incorporation by DNA polymerase. B) Comparison of all four dye-phosphate labeled triphosphates with all four dye-phosphate labeled tetraphosphates. Tetraphosphates are upto 50 fold better substrates than the corresponding triphosphates.

Procedure for a homogeneous 2-color SNP detection Assay

The procedure for carrying out a 2-color SNP analysis assay using genomic DNA PCR products to identify the homo- and heterozygous SNPs correctly involves the following steps:

a) the genomic DNA of interest for SNP analysis is first PCR amplified using AmpliTaq Gold with GeneAmp (Applied Biosystems) using the following reaction conditions. The typical PCR reaction contain

a. PCR reaction	1 x
10 X Reaction buffer	10 µL
25 mM $MgCl_2$	10 µL
2.5 mM dNTP each	8 µL
10 uM PCR forward primer	4 µL
10 uM PCR reverse primer	4 µL
AmpliTaq Gold 5U/µL	0.5 µL

25 ng/μL gDNA 2 μL
MilliQ water 61.5 μL
Total 100 μL

b. Thermal cycling

Denature 93 ^{0}C, 10 min
40 cycles 94 ^{0}C, 15 sec
53 ^{0}C, 20 sec
72 ^{0}C, 60 sec
Final extension 72 ^{0}C, 7 min

PCR product characteristics (size and purity) were verified by agarose gel and quantified by Picogreen assay. Although not necessary, the PCR product may be cleaned using the quick EXO-SAP treatment or other cleaning methods.

b) the primer was designed based on the site of the SNP interrogation. Thus, the designed primer has a non-hydrolyzable phosphodiester linkage at the 3'-end of the SNP site. The primer was annealed to the PCR product using the following conditions;

1. Preannealing SNP primer to PCR product
   This is carried out in MJR 96/384-well hard shell plate.

PCR product (about 10 ng/μL, 350 bp)	2 μL
SNP Primer (about 50 μM)	0.5 μL
ExoSAP-IT (USB Corp.)	0.5 μL
Hybridization Buffer (100 mM Tris, pH 8, 100 mM NaCl)	6 μL
MilliQ water	3 μL
Total	12 μL

Incubated at 37 ^{0}C for 15 min followed by denaturation at 95 ^{0}C for 5 min and then 0 ^{0}C.

2. Prepare SNP-SNAP Premix

Note: The nucleotide chosen for the premix should correspond to be the same as the expected genotype. For instances a SNP G/A requires a ddG4P (ddG-tetraphosphate) and ddA4P (ddA-tetraphosphate) combination.

Water	42 μL
250 mM HEPES, pH 8	14 μL
50 mM $MgCl_2$	14 μL
50 mM $MnCl_2$	1.4 μL
100 μM Ethyl Fluorescein-ddN4P	1.4 μL
100 μM Methyl Coumarin-ddN4P	1.4 μL
10 mM DTT	14 μL
1% Tween-20	1.4 μL
DNA Polymerase (Thermo Sequanase I)	0.8 μL
Exonuclease III	0.05 μL
Alkaline Phosphatase (BAP, SAP, CIP or others)	1.6μL

3. Combine 12 μL of step 1 with 23 μL of step 2 per reaction
4. Spin samples to remove air bubbles.

5. Leave at RT for 5 min.
6. Seal plate with a foil and incubate at 37 ^{0}C for 3 hrs.
7. Read on Tecan Ultra fluorescent plate reader using the appropriate filter sets for fluorescein and coumarin.
8. Include controls such as premix alone, template alone, SNP primer alone.

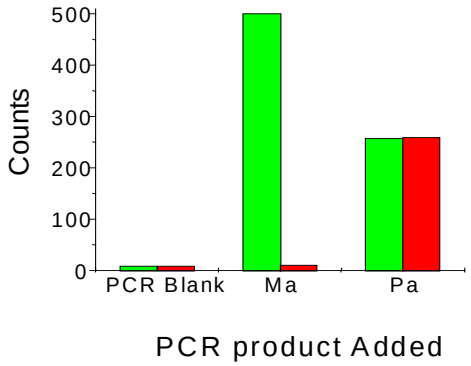
Results output as RFU can be normalized to maximal dye hydrolysis (nM) achieved with Snake venom phosphodiesterase (SVPDE) using more than one dye (1000, 100, 10nM) concentration (1U hydrolyses 1μM p-NTP per min at 25 C, pH 8.9).

Thus as shown above the protocol for the SNP analysis method is very simple and is shown below schemetically. After PCR in a 96 or 384 well plate, the SNP primers are annealed to the PCR products without any PCR product purification. A mixture of enzymes and nucleotides in appropriate buffer is then added and samples are incubated for 1-2h and simply read on a scanner or a plate reader. In a typical reaction S/N obtained is > 100.

Since the assay requires a small amount of template (1-2 fmol), PCR can be mutiplexed. Template from a 96plex PCR has been used to accrately score SNP's.

The power of this SNP analysis method is clearly demonstrated by the following two graphs. In the first case, samples from two individuals (Ma & Pa), one a homozygote and the other a heterozygote at NCBI422 locus, were analyzed. In the second graph, a panel of SNPs comprising 7 different loci and 12 individuals were analyzed. In all cases, just looking at the raw data the right calls were made. Results were later verified by sequencing.

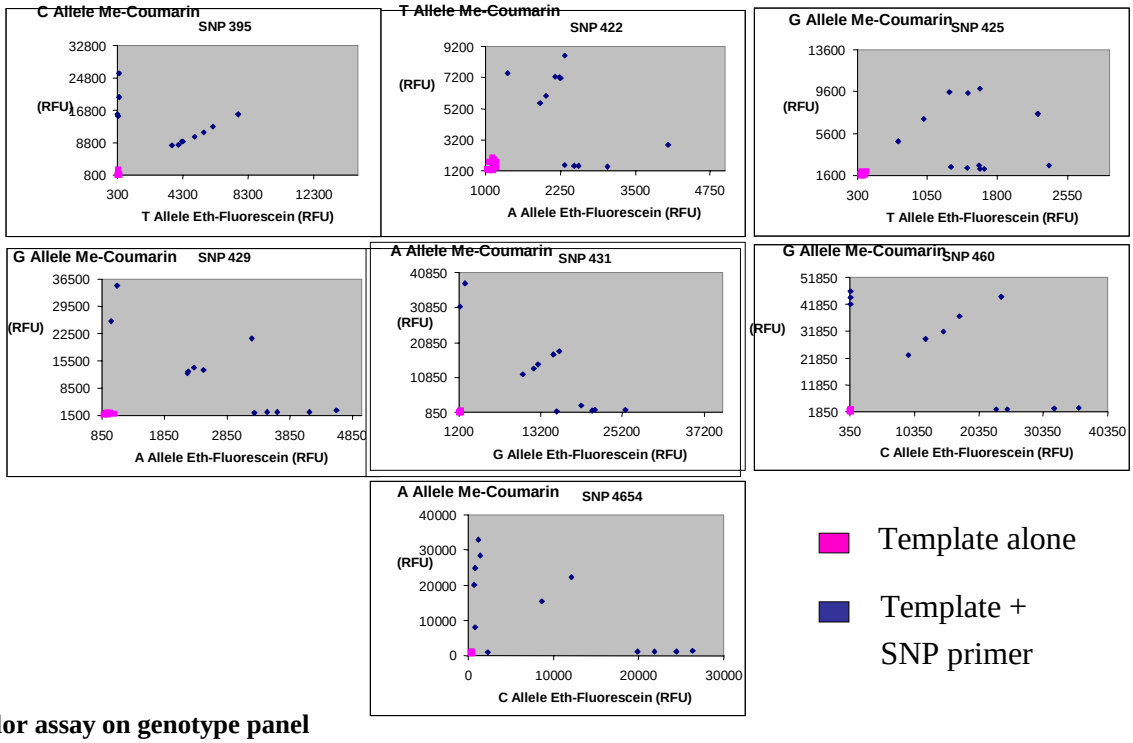
Results: SNP-SNAP



Analogs:
T-Fluorescein
A-DDAO

422SF primer
(for antisense strand)

SNP-SNAP on SNP Panel



2-color assay on genotype panel

A genotype panel covering 7 loci in 12 human genomic DNA samples (CORIELL INSTITUTE) was designed and verified using DNA sequencing. Grey areas indicate ambiguous genotypes and remain to be confirmed. They covered all possible genotype combinations i.e. A/G, C/T, G/T, A/C, C/G, T/A in PCR products ranging in size 254 - 394 bp long. PCR products were quantified by Picogreen assay (Molecular Probes) pre- and post-ExoSAPIT treatment and used in the SNP-SNAP assay. The yields of post-PCR product ranged from 1-20 ng/µL across the panel.

	NA 4660	NA 14661	NA 14663	NA 14665	NA 14672	NA 14682	NA 14683	NA 14696	NA 14698	NA 14700	NA 10835	NA 12248	Size (bp)
NCBI395	C/T	C/T	C	C/T	C	C/T	C/T	C	C/T	C/T	C/T	C	301
NCBI422	A/T	A/T	A/T	A/T	T	A	A	A	A/T	A	A	A/T	325
NCBI425	G/T	T	G/T	T	T	T	T	G/T	G/T	G/T	T	G/T	394
NCBI429	A/G	A/G	G	A/G	G	A	A	A	A/G	A	A	A/G	311
NCBI431	G	G	A/G	A/G	G	A/G	G	A/G	G	A/G	A	A	381
NCBI460	C	C/G	C	C	C/G	G	G	C	C/G	C/G	G	C/G	308
NCBI4654	A	A/C	A	A/C	A	C	C	C	C	C	A/C	A/C	254

Three replicates of the panel were made and tested. As shown above the SNP-dependent signal is well separated from the template-independent (no-template, primer alone). No incorrect (false) calls were observed. However, signal variation possibly due to variable amount of input PCR product was observed

Figure 3: HPLC-MS analysis of DDAO-labeled ddTTP (ddT3P-DDAO); (A) HPLC purity (B) MS Data (C) UV-VIS spectrum

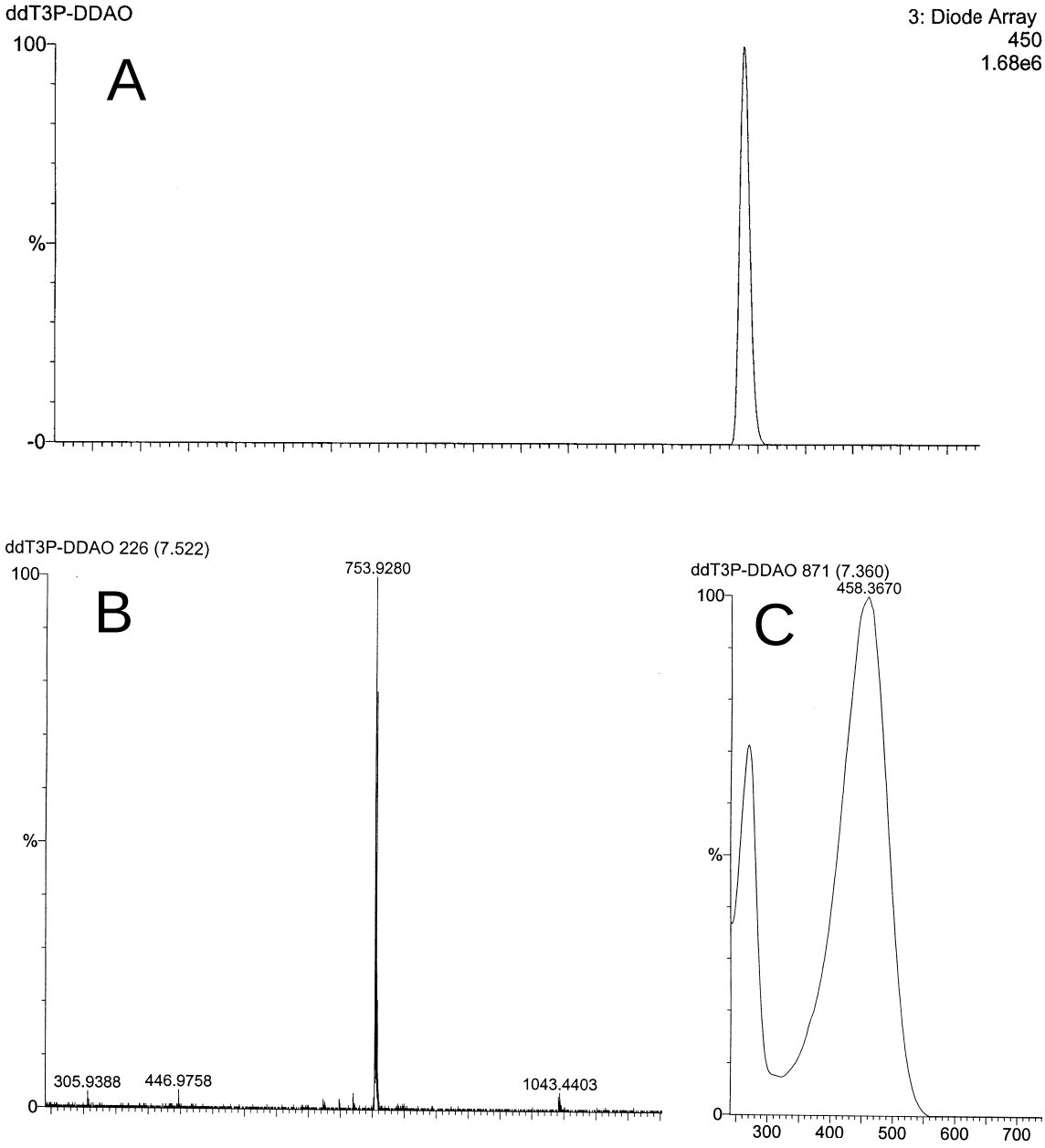


Figure 4: HPLC-MS analysis of DDAO-labeled dideoxythymidine-5'-tetraphosphate (ddT4P-DDAO); (A) HPLC purity (B) MS Data (C) UV-VIS spectrum

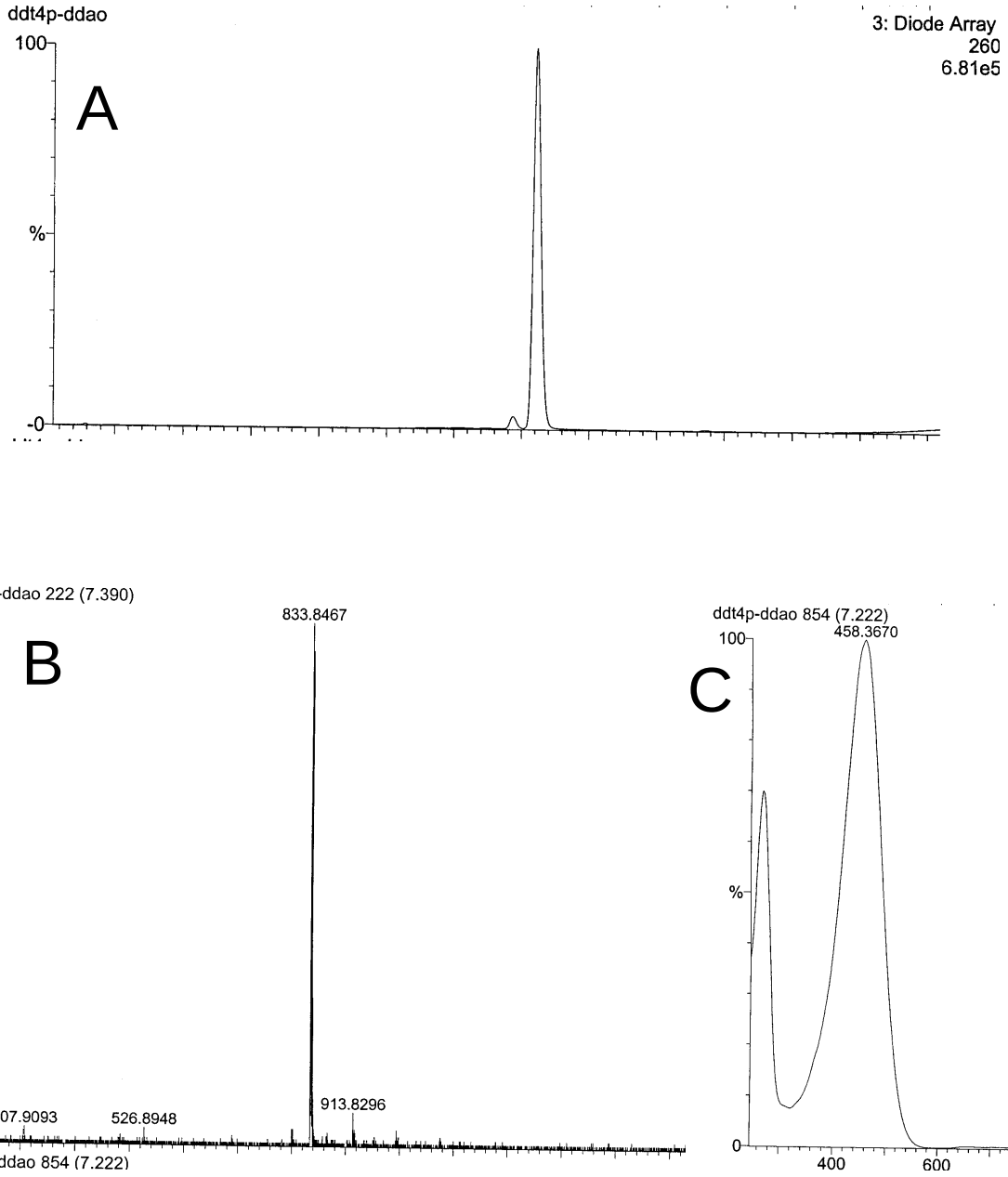


Figure 5: HPLC-MS analysis of DDAO-labeled dideoxythymidine-5'-pentaphosphate (ddT5P-DDAO); (A) HPLC purity (B) MS Data (C) UV-VIS spectrum

ddT5P-DDAO

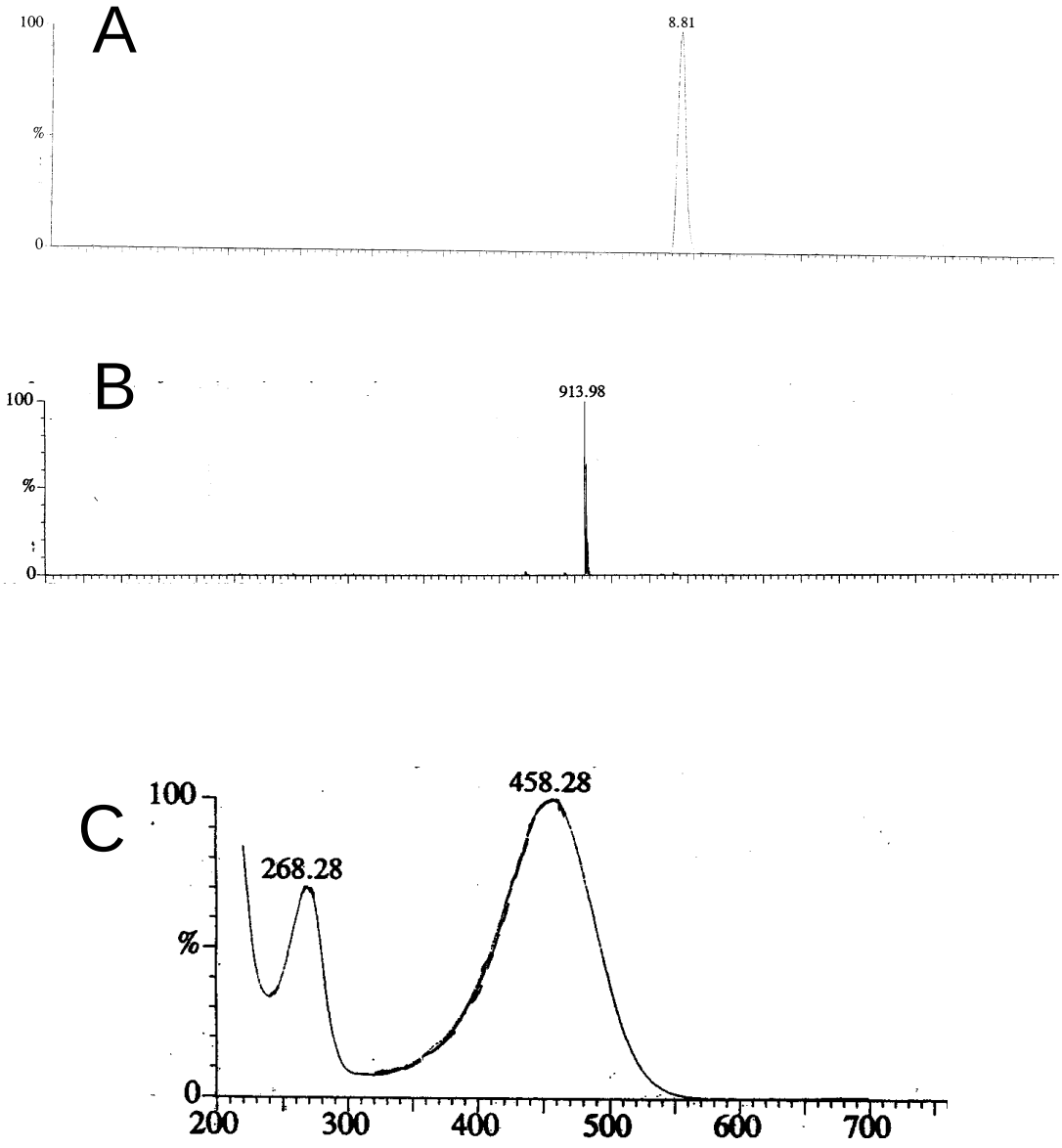


Figure 6: Representative ^{31}P-NMR spectrum of dye labeled triphosphate

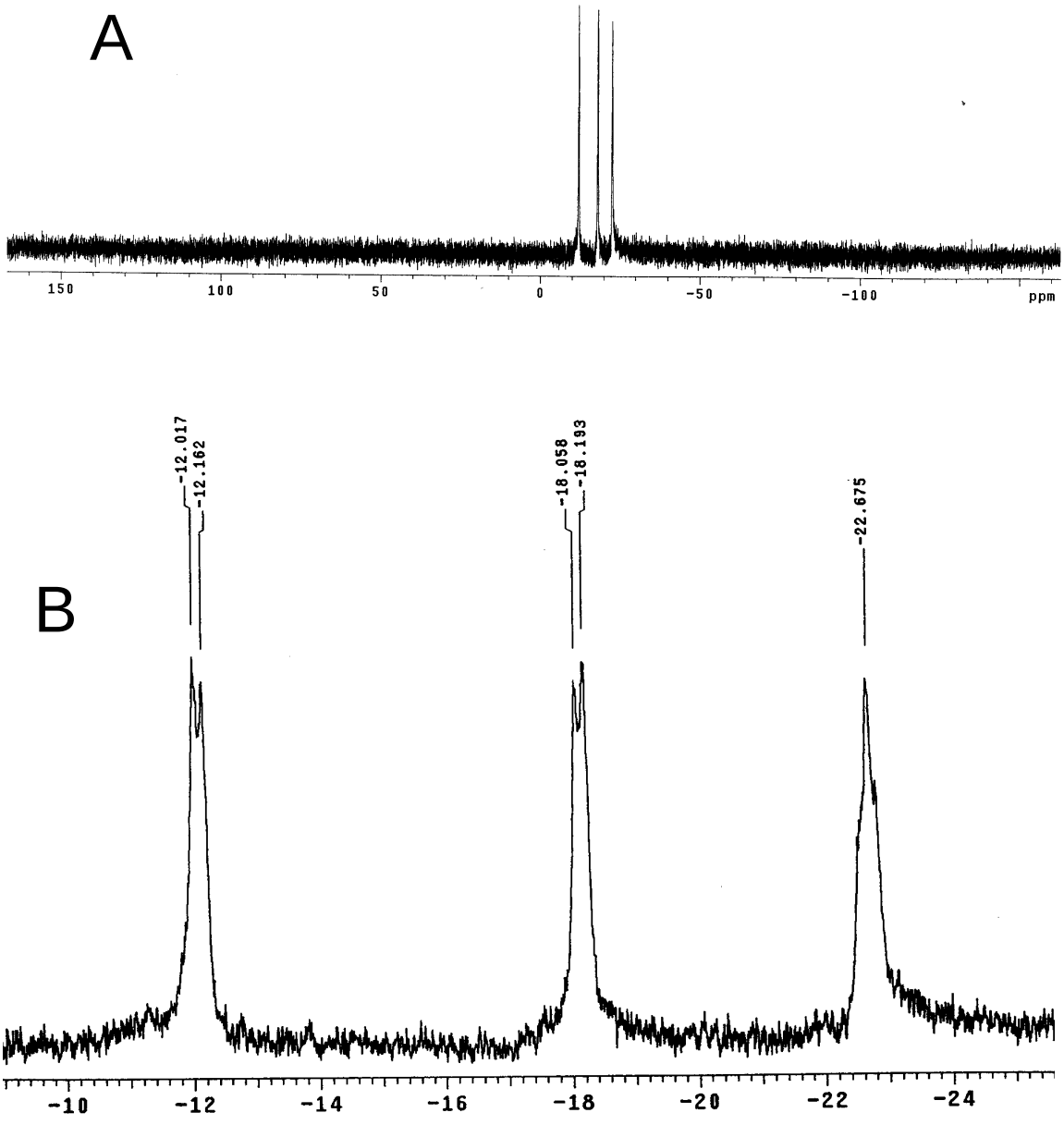


Figure 7: Representative ^{31}P-NMR spectrum of dye labeled tetraphosphate