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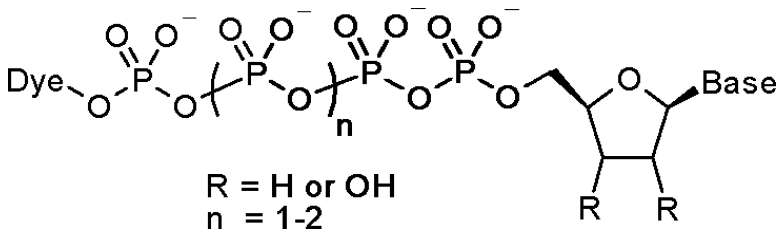

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Terminal Phosphate-Labeled Nucleotides with Improved Substrate Properties for Homogeneous Nucleic Acid Assays

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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

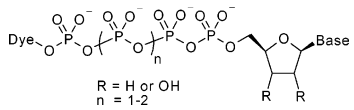


Figure 1. General structure of terminal phosphate-labeled nucleotides.

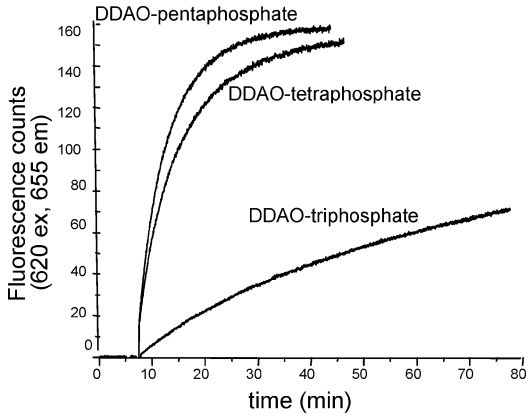
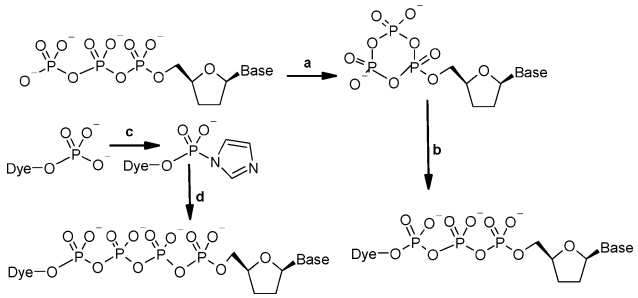


Figure 2. Depending on the DNA polymerase, nucleotide base, and reaction conditions, labeled tetra- and pentaphosphates give rates that are 10−50 times faster than those obtained with labeled triphosphates. Assays using *Taq* DNA polymerase at 42° with normal triphosphates give a rate of nucleotide addition 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

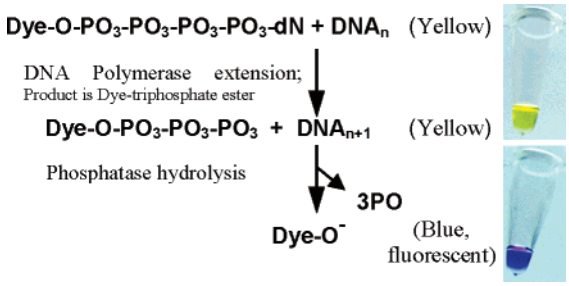

Figure 3. Combination of polymerase and phosphatase can result in homogeneous assays using suitable dyes. Suitable dyes are ones that change spectral characteristics between ester and anion forms.

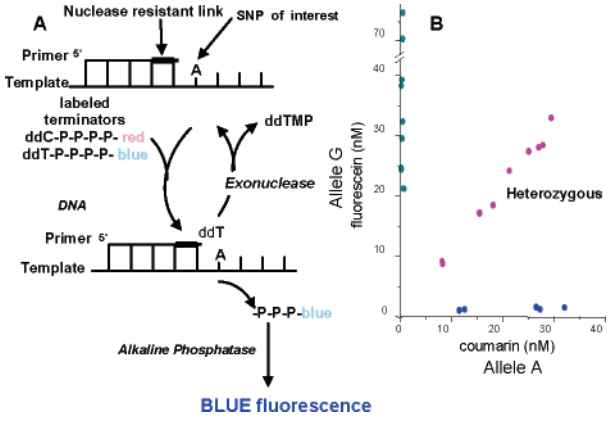

Figure 4. (A) An amplifying, homogeneous assay scheme for SNP typing using terminal phosphate-labeled nucleotides. Assays can contain two or four different dye-labeled nucleotides. (B) Assays for human SNP TSC0000431 using DNA from 12 different individuals.

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.

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