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ARTICLE TYPE

# Amplification of Fluorescent DNA through Enzymatic Incorporation of a Highly Emissive Deoxyguanosine Analogue†

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**A highly emissive thio-analogue of deoxyguanosine triphosphate, thdGTP, was synthesized and enzymatically incorporated into DNA through primer extension and PCR amplification. The straightforward amplification of thdG-labeled DNA by natural polymerases by simple addition of thdGTP to the standard PCR mix was demonstrated. The present results will facilitate the broad application of long fluorescent DNA.**

Fluorescent probes are powerful and indispensable tools with which to detect and monitor biomolecules. Their development combined with fluorescence spectroscopy techniques[1] is opening up new realm of research, and many biological phenomena have been understood by tracking fluorescent signals in living systems.[2] For nucleic acids, the development of fluorescent nucleobase analogues has become an important challenge since native nucleic acids are practically non-emissive.[3] It also has great significance in the viewpoint of the expansion of an artificial genetic molecule with diverse functionality. In this context, many researchers are endeavoring to design and synthesis fluorescent nucleoside surrogates that satisfy versatile photophysical properties and isomorphicity. Recently, Tor and co-workers developed isomorphic fluorescent RNA nucleosides that are characterized by the electronic and structural resemblance to the native nucleosides and have very significant photophysical features, including visible-light emission and high quantum yield.[4] We have also synthesized a highly emissive deoxyguanosine analogue, **thdG** (see Figure 1 (a)), and demonstrated that it can be used to enable the direct visualization of transitions between the B- and Z-forms of DNA as a result of different π-stacking.[5] In the previous study, **thdG** was synthetically incorporated into the oligonucleotide strands by automated solid-phase synthesis and the application of phosphoramidite chemistry. To further expand the utility of **thdG**, we turned to enzymatic incorporation through the use of naturally occurring enzymes and replication systems.[6-9]

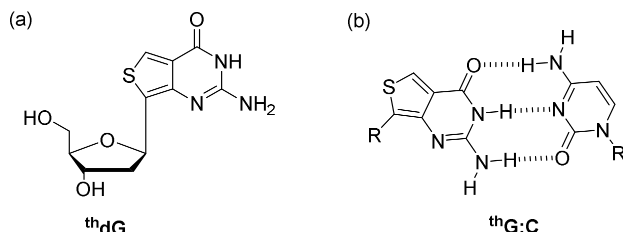
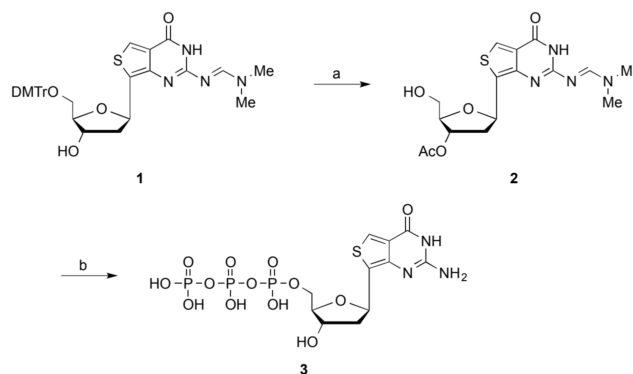



**Figure 1**. (a) **thdG** monomer. (b) Hydrogen bonding between **thdG** and dC (R=2′-deoxyribose).

Herein, we report the synthesis of a fluorescent nucleoside triphosphate, **thdGTP**, and its enzymatic incorporation into DNA through primer extension and PCR amplification. This study demonstrates that highly emissive **thdGTP** can be recognized and amplified in place of natural dGTP and that the preparation of long fluorescent DNA is feasible using enzymatic approaches and natural DNA polymerases.

The synthesis of **thdGTP** was performed by following published procedures for generating **thdG**[5] and triphosphate nucleoside derivatives[9] (Scheme 1). The protected guanosine mimic *O*5′-dimethoxytrityl-*N*2-DMF-2-aminothieno[3,4-*d*] pyrimidine deoxynucleoside was synthesized from commercially available methyl 4-aminothiophene-3-carboxylate hydrochloride. The 3′-hydroxyl group was protected by acetoxylation, and the dimethoxytrityl protection of the 5′-hydroxyl group was removed in dichloroacetic acid. The 5′-hydroxyl group was then phosphorylated with 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one and bis(tributylammonium) pyrophosphate following oxidization by $I_2$ solution, and the desired **thdGTP** was isolated by HPLC.




**Scheme 1**. Synthesis of **thdGTP**. Reagents and conditions: (a) (i) acetic anhydride, pyridine; (ii) dichloroacetic acid, DCM, 0 °C, 51%; (b) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, dioxane, pyridine, tri-*n*-butylamine, bis(tri-*n*-butylammonium)pyrophosphate, DMF, then $I_2$/pyridine/$H_2O$, 39%.

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Primer extension involving $^{th}$**dGTP** incorporation was examined by using the 3′→5′ exonuclease-proficient Klenow fragment, a FAM-labeled 10-mer primer, and a 17-mer DNA template **1**–**3** containing several cytosine residues in the extension area (see Figure S3). Cytosine residues at the 11th, 14th, or 17th position of the templates served as the paired base for $^{th}$**dG** (Figure 2a). These primer extension assays were analyzed by determining the length of the extension products using gel electrophoresis. To our delight, primer extension including $^{th}$**dGTP** incorporation opposite to C gave full-length products. Furthermore, primer extension assays with increasing numbers of $^{th}$**dGTP** incorporation positions also proceeded as well as with natural dGTP. To verify the incorporation of $^{th}$**dGTP** in the DNA, primer extension experiments were conducted with the 10-mer primer without FAM labeling; under these conditions, blue bands in the unstained gel were only observed in the lanes loaded with DNA incorporating $^{th}$**dGTP** (Figure 2b).

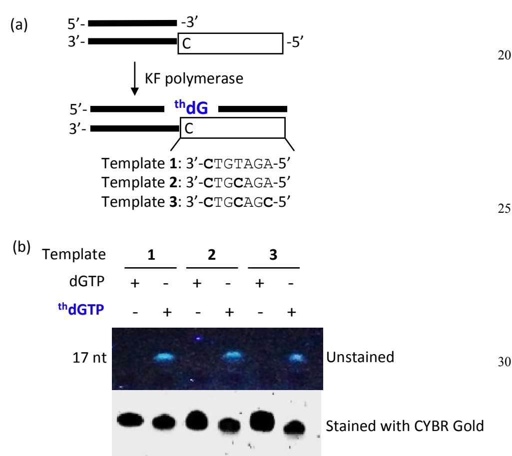



**Figure 2**. (a) Primer extension experiments with 17-mer templates **1**–**3**, in the presence of either natural dGTP or $^{th}$**dGTP** and three dNTPs (dATP, dTTP, and dCTP). (b) Analyses by denaturing gel electrophoresis of primer-extended products using primer without FAM labeling.

We conducted PCR amplification of the template containing only one cytosine in the amplification area with various polymerases, using $^{th}$**dGTP** instead of natural dGTP (Figure 3a). A 56-mer single-stranded DNA template was used together with forward and reverse primers that yield a product of the same length as the template. Throughout all of the experiments conducted in this study, the following PCR cycle was employed: 98 °C for 2 min, 95 °C for 15 sec, 53 °C for 30 sec, and 68 °C for 10 sec. After 40 cycles of PCR, the products were analyzed by native polyacrylamide gel electrophoresis (Figure 3b). KOD –Plus-, One*Taq*, and Deep Vent (exo–) polymerases all gave the full-length products. However, Deep Vent (exo+) polymerase gave only low amounts of product. It seems that 3′→5′ exonuclease-deficient DNA polymerase is more effective at incorporating $^{th}$**dG**. It is interesting to note that in addition to Deep Vent (exo–), which is a 3′→5′ exonuclease-deficient DNA polymerase, a high proof-reading polymerase, KOD -Plus-, could also incorporate $^{th}$**dGTP** in place of dGTP.

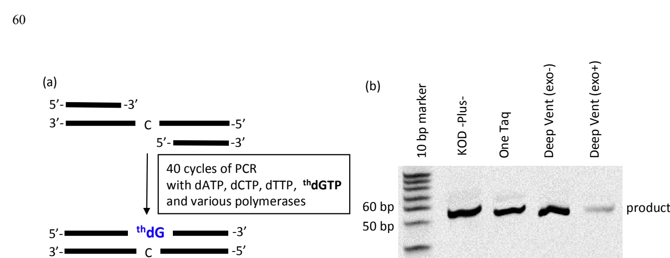
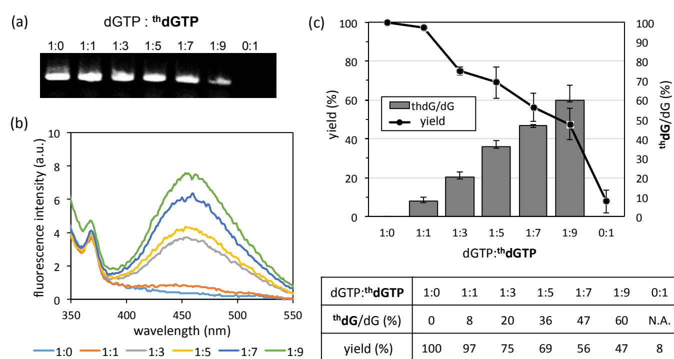



**Figure 3**. (a) Scheme for PCR amplification experiments using 56-mer templates, in the presence of $^{th}$**dGTP** with three dNTPs (dATP, dTTP, and dCTP). (b) Analyses by native PAGE of PCR-amplified products with KOD -Plus-, One*Taq*, Deep Vent (exo–), or Deep Vent (exo+).

These results inspired us to investigate the efficiency of PCR amplification of much longer templates that contain more cytosines as paired bases for $^{th}$**dG**. We first conducted a 338-mer PCR amplification with pET28a plasmid and KOD -Plus- polymerase, using $^{th}$**dGTP** instead of natural dGTP. However, the desired amplified product was not observed after fractionation by agarose gel electrophoresis, and no products were observed even after a much longer extension time (20 min). This result suggests that proximal $^{th}$**dGTP** residues are difficult to incorporate consecutively. We therefore conducted PCR amplification using a mixture of dGTP and $^{th}$**dGTP**. Upon increasing the proportion of $^{th}$**dGTP** gradually from 1:1 to 1:9, fluorescent labeling of the long DNA construct was achieved, and PCR products containing $^{th}$**dGTP** were obtained. The amplified products were fractionated and analyzed by agarose gel electrophoresis. After residual dNTPs were removed using a PCR purification kit (Sigma–Aldrich), fluorescence spectra were obtained for each DNA solution (Figure 4b).




| dGTP:$^{th}$dGTP | 1:0 | 1:1 | 1:3 | 1:5 | 1:7 | 1:9 | 0:1 |
|---|---|---|---|---|---|---|---|
| $^{th}$dG/dG (%) | 0 | 8 | 20 | 36 | 47 | 60 | N.A. |
| yield (%) | 100 | 97 | 75 | 69 | 56 | 47 | 8 |

**Figure 4.** (a) Analyses by agarose gel electrophoresis of amplified 338 bp products. PCR amplification was conducted with dCTP, dTTP, and dATP (200 μM each), and a combined total of 200 μM dGTP and $^{th}$**dGTP** mixture (1:0, 1:1, 1:3, 1:5, 1:7, 1:9, and 0:1). (b) Fluorescence spectra of purified DNA solutions obtained as described in (a) from dGTP/$^{th}$**dGTP** nucleotide ratios. All samples contained 3 μM DNA. Excitation wavelength was 325 nm. (c) Relationship between the dGTP/$^{th}$**dGTP** ratio and extent of $^{th}$**dG** incorporation and the yield of amplified products. Average of three runs.

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It was found that, as expected, the intensity of fluorescence of the purified DNA solution increased with the proportion of **$^{th}$dGTP**. This indicated that a substantial amount of **$^{th}$dGTP** was incorporated during PCR. To gain a more detailed understanding, the amplified DNA products were hydrolyzed and the respective amounts of constituent nucleosides were evaluated quantitatively. The amount of **$^{th}$dG** was deduced using a standard HPLC chart obtained by injecting equimolar amounts of nucleosides including **$^{th}$dG**. After enzymatic hydrolysis, HPLC analyses indicated that the ratio of **$^{th}$dG** to dG in the amplified products increased with the **$^{th}$dGTP**/dGTP ratio as shown in Figure 4c, although the yield of the amplification product diminished. This result implies that the ratio of **$^{th}$dGTP** to dGTP should be adjusted according to the intended purpose. Encouraged by these results, we established a simple enzymatic method with which to incorporate **$^{th}$dG** into DNA by simply adding **$^{th}$dGTP** (5 equiv) into the standard PCR reaction mixture. To investigate the scope of the system with respect to the amplified products, we conducted PCR amplification of 298-, 480-, and 761-mer DNA using pGEM or pUC18 plasmids with other sets of primers. As a result, fluorescent products of the expected length were obtained that were labeled by **$^{th}$dG** (Figure 5).

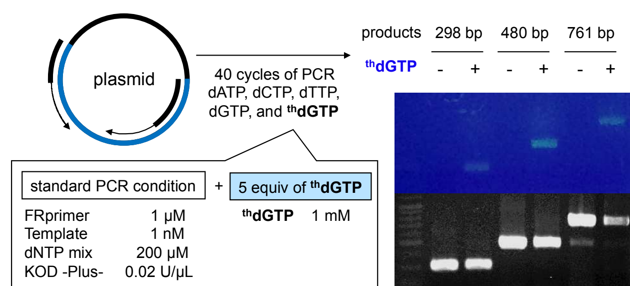


**Figure 5.** Simple **$^{th}$dG** labeling for long fluorescent DNA. Analyses by agarose gel electrophoresis of PCR-amplified products; **$^{th}$dGTP** (5 equiv, 1 mM) was added to 200 μM dNTPs (dCTP, dTTP, dATP, and dGTP) and standard PCR reaction mixture. Photograph taken (top) under 254 nm irradiation before staining, and (bottom) after staining with ethidium bromide.

In conclusion, we have synthesized a visible fluorescent nucleoside triphosphate, **$^{th}$dGTP**, and incorporated it into DNA through primer extension and PCR amplification. **$^{th}$dGTP** can be incorporated into DNA with only slightly lower efficiency than natural substrate dGTP, and straightforward addition of **$^{th}$dGTP** into the standard PCR mixture gives emissive **$^{th}$dG**-labeled DNA strands that can be observed under UV irradiation with the naked eye. We expect that this methodology, which keeps the inherent structure of DNA intact, will facilitate the application of long fluorescent DNA in areas such as the construction of fluorescent DNA nanostructures. Furthermore, these results raise the intriguing possibility that **$^{th}$dG** could be incorporated into living cells as a fluorescent probe.[10,11]

## Notes and references

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†Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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