

# PCR incorporation of modified dNTPs: the substrate properties of biotinylated dNTPs

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Modified dNTPs are a convenient tool for the enzymatic introduction of functional groups into a nucleic acid target of interest. One major benefit of enzymatic functionalization is uniform distribution of modifications across a long target sequence. In comparison, using modified oligonucleotides is an appropriate choice when shorter sequences modified in discrete locations are desired. Nick translation and random labeling are the most commonly used methods for modified dNTP incorporation. These methods are generally applied to complex nucleic acid mixtures (1,2). When a specific primer pair is used in combination with DNA polymerase, a functionalized double stranded DNA copy of a defined region is generated.

Although modified dNTPs can be employed in a number of different enzymatic protocols, the position and type of attachment have a strong influence on DNA polymerase efficiency. Extensive investigation has revealed that modifications to the major groove of the nucleobase allow for the best incorporation efficiency with the 5-position of pyrimidines and the 7-position of purines being the optimal site (1). The flexibility of the linker arm attaching the modification can also influence nucleotide utilization, with rigid, linear linkers providing the strongest dNTP substrate properties. Linker arm length also plays a role in modified dNTP incorporation. Modified dNTPs with shorter linker arms (i.e., biotin-4-dUTP) are better substrates than nucleotides with longer linker arms (i.e., biotin-11-dUTP or biotin-14-dUTP) (2). With the knowledge of linker position and length, functional groups can be introduced for each of the four dNTPs. However, often only one of the four modified dNTPs is offered commercially, with 5-substituted dUTP analogs being the most readily available.

There are a number of considerations for efficient amplicon formation when employing modified dNTPs. While Taq DNA polymerase is traditionally used, in some instances members of the B-family of polymerases provide greater PCR efficiency. Furthermore, complete substitution of a modified dNTP for its natural counterpart causes inhibition of PCR, resulting in low and often undetectable product formation. This is due to the compounding effect of a lower efficiency of nucleotide incorporation over multiple cycles. In order

to achieve a high degree of functionalization without compromising yield, the percentage of substitution of a modified dNTP for its natural counterpart needs to be experimentally determined. Herein we will describe an approach for modified dNTP incorporation in PCR, using biotinylated dNTPs. The goal is to label the resultant amplicon at high density with biotin using 5-modified biotin-16-aminoallyl dUTP and dCTP.

## Materials and Methods:

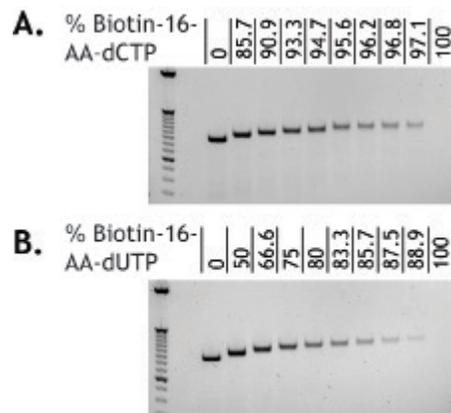
Biotinylated nucleotides were prepared by conjugation of 5-aminoallyl-dCTP and 5-aminoallyl-dUTP (TriLink BioTechnologies, Cat. no. N-2048 and N-2049) with a biotin-containing linker to prepare biotin-16-AA-dCTP and biotin-16-AA-dUTP (TriLink BioTechnologies, Cat. no. N-5002 and N-5001), respectively. PCR conditions were performed as described in *Enzymatic Incorporation of Biotin-16-AA-dNTPs*, available at [trilinkbiotech.com/work/BiotindNTPs.pdf](http://trilinkbiotech.com/work/BiotindNTPs.pdf).

## Results and Discussion:

The high affinity of streptavidin for the biotin ligand is one of the strongest and most widely utilized interactions in biology. The strength and specificity of this interaction has been exploited in many biological applications, including secondary label introduction and affinity isolation. In PCR, biotinylated dNTPs with shorter linker arms (i.e., biotin-4-dUTP) serve as better DNA polymerase substrates. However, biotinylated dNTPs with longer linker arms (i.e., biotin-11-dUTP or biotin-14-dUTP) are more commonly used because they improve detection by streptavidin-biotin complex formation (2). Focusing on biotinylated dNTPs suitable for strong post-amplification detection, the extent of biotin-16-AA-dNTP substitution was investigated.

Prior to conducting extensive modified dNTP optimization studies, a good starting point is to test 0%, 25%, 50%, 75%, and 100% modified nucleotide substitution for its natural counterpart. As expected, amplicon formation was not detectable for 100% biotinylated dNTP substitution. Interestingly, for both biotin-16-AA-dNTP analogs, strong amplicon formation was evident at 75% substitution. To understand where between 75% and 100% substitution amplicon yield begins to be compromised, a second experiment was performed. For biotin-16-AA-dUTP, yield de-

creased quickly above 75% substitution, while for biotin-16-AA-dCTP, yield decreased at a much higher substitution of ~90%. Further studies sought to better understand this difference.



**Figure 1.** Biotinylated dNTP incorporation in PCR. Agarose gel analysis of PCR conditions where the percentage of (A) biotin-16-AA-dCTP and (B) biotin-16-AA-dUTP was titrated between 0% and 100%. Percentages indicate the percentage of biotinylated nucleotide substitution for its natural counterpart.

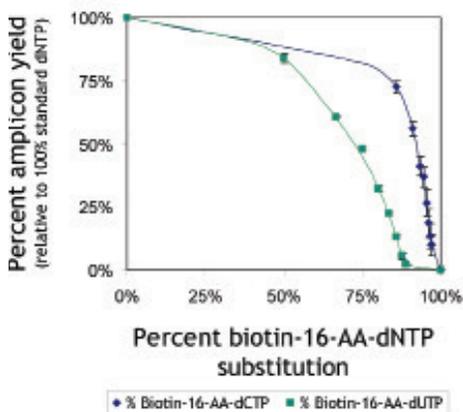
A more thorough investigation of biotin-16-AA-dNTP substitution in PCR was performed (Figure 1). While the percentage of biotin-16-AA-dNTP substitution and its effect on amplicon yield varied for the dCTP and dUTP analogs, a few general trends were evident. First, as the percentage of biotinylated nucleotide increased, a decrease in amplicon mobility was observed. As described, this mobility change is due to the large bulk introduced by the biotin side chain (3). Second, complete substitution of the biotinylated dNTP for its natural counterpart caused complete inhibition of the reaction as no product formation was detected. In addition, the detailed studies confirmed the observation that Taq DNA polymerase incorporated biotin-16-AA-dCTP (Figure 1A) with greater efficiency than biotin-16-AA-dUTP (Figure 1B).

Quantification of these results (Figure 2) revealed that although amplicon formation was possible at 89% substitution with biotin-16-AA-dUTP, the use of biotin-16-AA-dCTP allowed for up to 97% substitution. While high substitutions are achievable, yield was significantly compromised. To determine a composition of biotinylated dNTP that will

allow for robust labeling without a significant reduction in yield, compositions that provided a ~50% reduction in amplicon yield were identified. For biotin-16-AA-dUTP, a 50% reduction in amplicon yield was seen at ~75% substitution, whereas for biotin-16-AA-dCTP, a 50% reduction was seen at ~92% substitution. While the extent of biotin labeling achieved is significantly greater than the recommended range for streptavidin complex formation (3-10%) (2), it is noteworthy that higher-density biotin labeling is attainable. These findings also highlight major differences in the extent of biotin labeling, depending on which of the pyrimidine analogs are employed. The strong performance of biotin-16-AA-dCTP is of particular interest since many researchers commonly employ dUTP analogs in labeling schemes.

#### Outlook:

It is a common misconception that complete substitution of a modified dNTP for its natural counterpart will be successful. However, optimizations—such as those outlined herein—may be necessary to achieve robust PCR performance. Furthermore, the unexpected benefit of employing biotinylated dCTP is significant, particularly because of the greater abundance of dUTP analogs with modifications at the 5-position of the nucleobase.



**Figure 2.** PCR amplicon yields for biotin dNTP incorporation. Data represent average amplicon yields from triplicate experiments, with error bar representing the standard error of the mean.

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