

Robust PCR amplification of GC-rich targets with Hot Start 7-deaza-dGTP

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PCR is a well-known and effective tool for the amplification of DNA targets of interest. When DNA targets high in GC content are amplified, PCR product formation can often be compromised by inadequate strand separation and the propensity for complex secondary structure formation. The inability of the DNA polymerase to negotiate through regions of strong secondary structure, such as hairpins, often results in the formation of truncated PCR products. In addition, mis-priming amplification products are prevalent when primers are high in GC content (1). Despite the inherent challenges, detection of GC-rich sequences is becoming increasingly important for the molecular diagnosis of inherited diseases (2).

Traditional approaches, such as specialized DNA polymerases and Hot Start technologies, have the potential to improve some GC-rich PCR assays provided that secondary structure formation is minimal (1). Many unique methods have been developed to enable amplification of sequences high in GC content, including the addition of organic molecules, the modification of thermal cycling protocols, and the use of modified dNTPs. Although each of the above-mentioned methods provides an improvement in GC-rich target amplification, various combinations of these methods are often required to obtain the desired specificity.

One of the most noteworthy modified dNTPs used to amplify GC-rich targets is 7-deaza-dGTP, a dGTP analog that lacks nitrogen at the 7 position of the purine ring. The use of this modified nucleotide blocks Hoogsteen bond formation without interfering with normal Watson-Crick base pairing, reducing the probability that secondary structures, such as G quadruplexes, will form (1). To further improve GC-rich target amplification, a Hot Start version of 7-deaza-dGTP has recently been developed. This new dNTP analog is an elegant fusion of the secondary structure reducing nucleotide analog 7-deaza-dGTP and TriLink BioTechnologies' CleanAmp™ dNTP Hot Start technology. The CleanAmp™ dNTP technology employs a thermolabile modification group at the 3'-hydroxyl that blocks dNTP incorporation at low temperatures and when released at higher thermal cycling temperatures produces a suitable dNTP substrate for DNA polymerase incorporation (3,4). CleanAmp™ 7-deaza-dGTP will be described herein as an effective and simple method that greatly improves and in

many cases enables specific amplification of GC-rich regions of varying complexity.

Materials and Methods:

PCR protocols for the amplification of targets with varying percent GC content using several dNTP combinations (Figure 1) consisted of 1x PCR buffer (supplemented with MgCl₂ to 2.5 mM; Invitrogen), 1.25 U *Taq* DNA polymerase (Invitrogen), 0.1 μM BRAF primer pair, 0.2 μM all other primer pairs (TriLink BioTechnologies, Inc.), 0.2 mM dNTPs (standard dNTPs, New England Biolabs; CleanAmp™ dNTPs, TriLink BioTechnologies, Inc., Cat. no. N-9501 or N-9505), and 5 ng human genomic DNA as template (Promega). Reactions with 7-deaza-dGTP

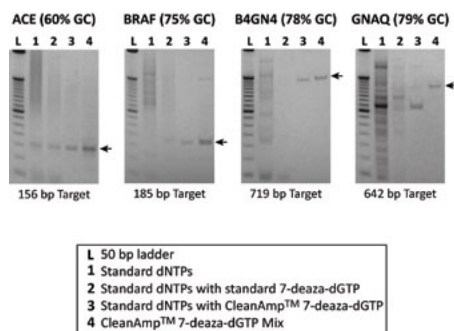


Figure 1. PCR amplification of targets with varying percent GC content: Evaluation of the effect of 7-deaza-dGTP for reactions containing various combinations of standard and CleanAmp™ dNTPs, 5 ng of human genomic DNA and *Taq* DNA polymerase.

or CleanAmp™ 7-deaza-dGTP (both from TriLink BioTechnologies, Inc., Cat. no. N-2011 and N-9515, respectively) used a dNTP mixture of the following composition: 0.2 mM d(A,C,T)TP, 0.05 mM dGTP, and 0.15 mM 7-deaza-dGTP. All 25 μL reactions were performed in quadruplicate and run on a Bio-Rad Tetrad 2 thermal cycler with a thermal cycling protocol of 95°C (10 min); [95°C (40 sec), X°C (1 sec), 72°C (1 min)] 35 cycles; 72°C (7 min) where X (annealing temperature) varied depending on the target (ACE 66°C; BRAF 64°C; B4GN4 57°C; GNAQ 66°C). A one second annealing time was found to be important for specificity and was used throughout these studies. The GNAQ target was amplified for 40 cycles. After PCR, 20 μL of each sample was analyzed by agarose gel electrophoresis. The primer sequences used in these studies were: ACE (156 bp; 60% GC-rich: 5'-GGGATGGTTGCCAGGCTGGA-3' and 5'-TGATGGGTGCCGTGGTCTCTCT-3') (1);

BRAF (185 bp; 75% GC-rich: 5'-CTCGGT-TATAAGATGGCGGCGCTGA-3' and 5'-AGTC-GGGAGGGCGGCAGGGT-3') (1); B4GN4 (719 bp; 78% GC-rich: 5'-GCCTGGAGCCCACCGAG-3' and 5'-CGTGCCTGCCAGTCA-3') (5), and GNAQ (642 bp; 79% GC-rich: 5'-CCTCCTCCCGGGAACAGGC-3' and 5'-CCGAGGTGCGGGAG-CAGTAG-3') (1).

The experimental conditions for Figure 2 were similar to those listed for Figure 1 with the following additions: only CleanAmp™ versions of dNTPs and 7-deaza-dGTP were used (CleanAmp™ 7-deaza-dGTP Mix, TriLink BioTechnologies Inc., Cat. no. N-9504); all primers were used at 0.2 μM concentration; human genomic DNA template was present at four concentrations (0.5, 5, 50, and 500 ng); the annealing temperatures (X°C) for AGT and GNB3 were 61°C and 55°C, respectively, and 35 thermal cycles were performed. The following additional primer sequences were used: AGT (242 bp; 61% GC-rich: 5'-CCT-GGAAGAGTCCCAGGCTGAGT-3' and 5'-GACAAAGACCAGAAGGAGCTGAGG-3') (1) and, GNB3 (92 bp; 61% GC-rich: 5'-CTCCCACGAGAGCATCATCTG-3' and 5'-GCAGTTGAAGTCGTCTAGCC-3') (1).

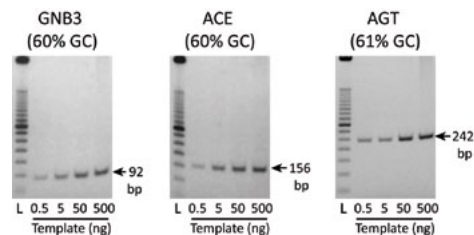


Figure 2. Detection of three targets with ~60% GC content using the CleanAmp™ 7-deaza-dGTP Mix: Targets were amplified with *Taq* DNA polymerase using a range of input human genomic DNA from 0.5 to 500 ng.

In the final experiments which explored the use of the CleanAmp™ 7-deaza-dGTP Mix with alternative DNA polymerases and different input template concentrations (Figure 3), methods were similar with these additions: GNAQ primers were used and the thermal cycling protocol described for Figure 1 above used a 66°C annealing temperature and 40 thermal cycles. For Figure 3A, 5 ng of human genomic DNA was amplified with the following three additional enzymes: a) DyNAzyme® II DNA polymerase (Finnzymes, 1.25 U, with 1X Optimized DyNAzyme® buffer supplemented

with $MgCl_2$ to 1.5 mM); b) *Pfu*(exo-) DNA Polymerase (Stratagene, 1.25 U, with 1x Cloned *Pfu* buffer supplemented with $MgSO_4$ to 2 mM), and c) DeepVent_RTM (exo-) DNA Polymerase (New England Biolabs, 1.25 U with 1x Thermopol reaction buffer supplemented with $MgSO_4$ to 2 mM). In Figure 3A, standard dNTPs were compared to CleanAmpTM 7-deaza-dGTP Mix. In Figure 3B, only CleanAmpTM analogs were used to amplify human genomic DNA (0.05 ng, 0.5 ng, 5 ng, and 50 ng) and *Pfu* (exo-) and DeepVent_RTM (exo-) were the DNA polymerases tested.

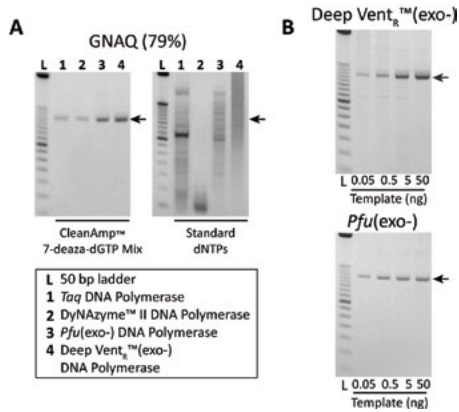


Figure 3. Optimization for the PCR amplification of the GNAQ target (79% GC): (A) Target amplification with standard dNTPs or the CleanAmpTM 7-deaza-dGTP Mix using a variety of DNA polymerases and 5 ng of human genomic DNA. (B) Sensitivity of amplification when the CleanAmpTM 7-deaza-dGTP Mix is used with *Pfu* (exo-) or DeepVent_RTM (exo-): Reactions included *Pfu* (exo-) or DeepVent_RTM (exo-) DNA polymerases, the CleanAmpTM 7-deaza-dGTP Mix, and human genomic DNA concentrations ranging from 0.05 to 50 ng.

Results and Discussion:

The importance of GC-rich nucleic acid sequence detection is marked by the need for successful amplification of these regions in the molecular diagnosis of inherited diseases such as Fragile X syndrome, Hirschsprung disease, and Congenital Central Hypoventilation syndrome. These diseases are caused by defects in the promoter, enhancer and control element regions of genes which all tend to be very GC-rich (2). Although there are many potential solutions for the amplification of GC-rich regions, none are optimal and therefore may require extensive optimization. To help address this growing need, we investigated the utility of a Hot Start version of 7-deaza-dGTP, CleanAmpTM 7-deaza-dGTP, as a means of improving amplification specificity.

To compare the effect of standard and Hot Start versions of 7-deaza-dGTP, several GC-rich targets were amplified with four different

combinations of nucleotides. The nucleotide combinations used in these studies were (1) standard dNTPs, (2) standard dNTPs with standard 7-deaza-dGTP, (3) standard dNTPs with CleanAmpTM 7-deaza-dGTP, and (4) CleanAmpTM dNTPs with CleanAmpTM 7-deaza-dGTP (CleanAmpTM 7-deaza-dGTP Mix). Reactions containing 7-deaza-dGTP used a 3:1 ratio to dGTP as described by McConlogue (6). Figure 1 shows the results of the PCR amplification of four GC-rich targets ranging from 60% to 79% GC content (1,5). Although results indicate that the inclusion of standard 7-deaza-dGTP to reactions with standard dNTPs improves the amplification of GC-rich targets, a further improvement in specificity and amplicon yield is seen when CleanAmpTM 7-deaza-dGTP is combined with standard dNTPs. However, when targets with higher GC content, such as GNAQ, are amplified, the desired amplicon may not form with high specificity. When CleanAmpTM 7-deaza-dGTP Mix is used, amplification yield is improved, and the correct amplicon is successfully formed for all four GC-rich targets. All the amplicons formed specifically, except for BRAF, which formed a small amount of off-target product. These results indicate that a combination of CleanAmpTM dNTPs with CleanAmpTM 7-deaza-dGTP functions well for routine GC-rich target amplification.

Although a set of sequences may have a similar GC percentage, their ease of amplification may vary due to the inherent complexity or denseness of the GC-rich regions within the sequence. To better understand differences in sequence complexity as they relate to GC-rich amplification, three targets with ~60% GC content (ACE, AGT and GNB3(1)) were evaluated (Figure 2). When the three targets were amplified with the four nucleotide combinations as described for Figure 1, varying results were seen across each of the combinations one to three, confirming a difference in GC content complexity (data not shown for AGT and GNB3). However, all three targets showed high specificity and adequate amplicon yield when amplified with nucleotide combination four, CleanAmpTM 7-deaza-dGTP Mix. The CleanAmpTM 7-deaza-dGTP Mix was then tested to determine the limit of detection of these sequences in PCR (Figure 2). This mixture allowed for successful amplification of the desired target over a wide range of template concentrations (0.5 to 500 ng).

For challenging targets with higher GC content, specificity remains high with the use of CleanAmpTM 7-deaza-dGTP Mix, but amplicon yield may be low. It was of interest to determine whether amplicon yield could be enhanced by the use of different DNA polymer-

ases. Results in Figure 3A demonstrate that the three new DNA polymerases examined specifically amplify the GNAQ target (79% GC) with the CleanAmpTM 7-deaza-dGTP Mix. In comparison, the amplification of this target with standard dNTPs was not possible by a simple change of DNA polymerase. Both *Pfu* (exo-) and DeepVent_RTM (exo-) showed significant improvement in amplicon yield when used in combination with CleanAmpTM 7-deaza-dGTP Mix. These two DNA polymerases were then used in further experiments to amplify GNAQ from input template concentrations between 0.05 and 50 ng (Figure 3B). A simple substitution of the DNA polymerase allowed for a significant improvement in the limit of detection for this challenging target.

Although GC-rich targets remain difficult to amplify, CleanAmpTM 7-deaza-dGTP greatly enhances amplification specificity. While CleanAmpTM dNTPs improve specificity in routine PCR, they were not a complete solution for the amplification of targets with high GC content. Similarly, the use of Hot Start DNA polymerases with 7-deaza-dGTP was not always effective. However, the combination of CleanAmpTM dNTPs with CleanAmpTM 7-deaza-dGTP (CleanAmpTM 7-deaza-dGTP Mix; TriLink BioTechnologies, Inc., Cat. no. N-9504) provides a simple and effective Hot Start solution for amplification of GC-rich targets and is a key addition to further refine the molecular diagnostics toolbox. To learn more about this product, please visit our website www.trilinkbiotech.com/cleanamp.

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