Chemical Variant of 7-deaza-dGTP for Improved GC-rich PCR Amplification

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Abstract

Background:
PCR amplification of nucleic acids is a fundamental technique used in many molecular biology laboratories. Despite its wide use, certain GC-rich regions of DNA, such as mycobacterial disease targets, still remain a challenge for amplification. Sequences high in GC content are associated with the formation of secondary structure, which prevents adequate strand separation and DNA polymerase amplification. As a consequence, mispriming is prominent, complicating specific product formation. In the area of molecular diagnosis of inheritable diseases, several assay modifications have been developed to improve the specificity of target amplification. Such approaches include specialized polymerases, Hot Start assays, addition of organic molecules, and thermal cycling alterations. However, as the GC content increases, the combination of two or three approaches may be required.

Methods:
Here, we show how 7-deaza-dGTP, a commonly used molecule to amplify GC-rich targets, can highly improve results when a thermolabile protecting group is incorporated at the 3´-hydroxyl. The presence of the protecting group blocks low temperature primer extension and only allows nucleotide incorporation at higher temperatures when the protecting group is removed, improving PCR specificity as a result.

Results:
This Hot Start version of 7-deaza-dGTP improves the amplification of targets containing moderate GC content (less than 70%). Results were further improved when a Hot Start version of all dNTPs was employed. The CleanAmp™ 7-deaza-dGTP Mix can amplify targets up to 84% GC-rich without the need for any additional additives or enzymes.

Conclusion:
The use of dNTPs modified with thermolabile chemistry simplifies GC-rich amplification and provides a valuable solution that can improve disease diagnosis.

Figure 1

Proposed activation mechanism of CleanAmp™ dNTPs

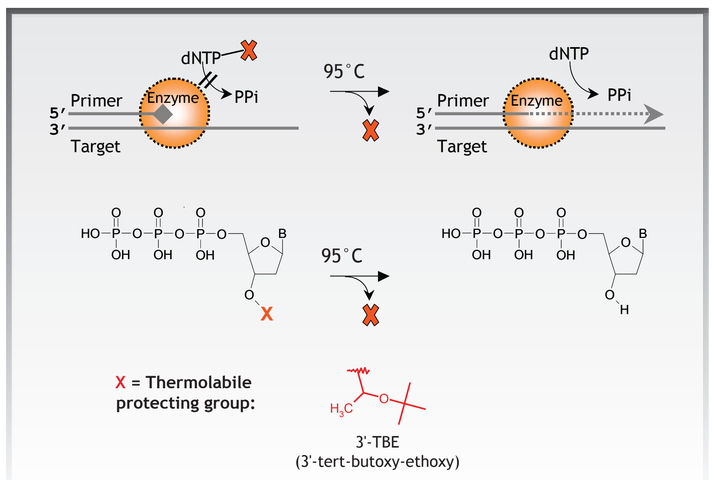
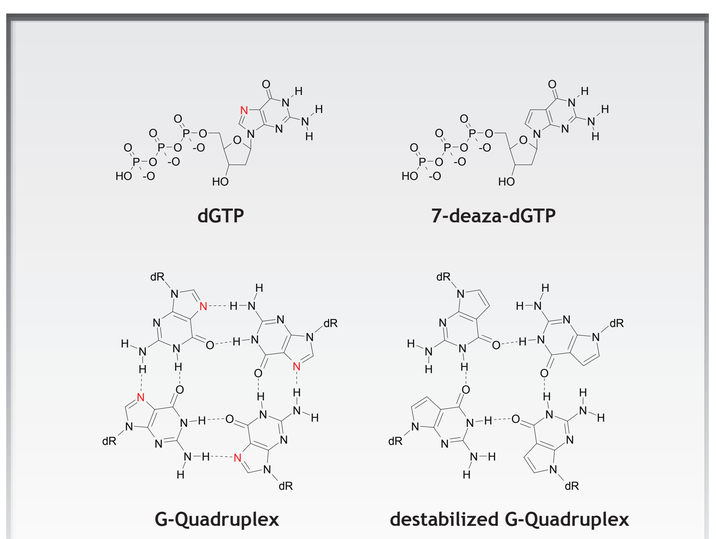


Figure 2

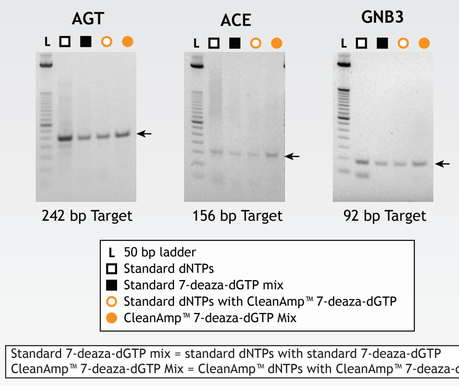
Reduction of GC-Rich secondary structure formations



The addition of the CleanAmp™ modification onto 7-deaza-dGTP could be applied to further improve GC-rich amplification.

Figure 3

Assessment of 7-deaza-dGTP for the amplification of targets with 60% GC Content



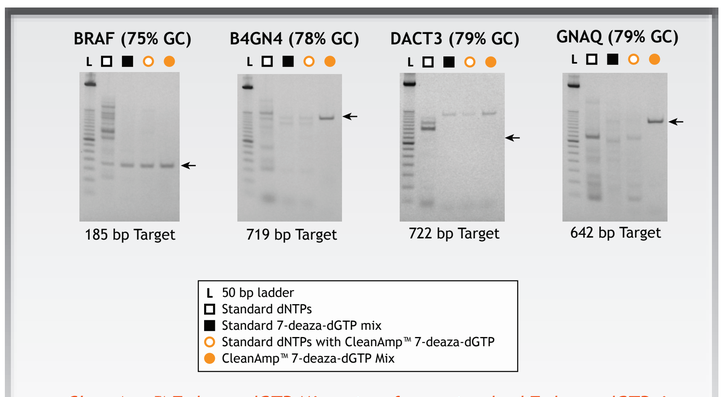
CleanAmp™ 7-deaza-dGTP Mix shows slightly improved performance with targets at lower GC content ~60%.

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$); Standard dNTPs = 0.2 mM d(A, C, G,T)TP; Reactions containing 7-deaza-dGTP contained 0.2 mM d(A, C, T,)TP (standard or CleanAmp™); 0.05 mM dGTP (standard or CleanAmp™) and 0.15 mM 7-deaza-dGTP (standard or CleanAmp™); Primer conc.: 0.2 µM (AGT, ACE, GNB3); 1.0 U *Taq* DNA polymerase; 0.2 ng/µL Human gDNA; 25 µL.

Thermal cycling conditions: 95˚C (10 min) [95˚C (40 sec), X˚C (1 sec), 72˚C (1 min)] 35X, 72˚C (7 min) (X= 61˚C (AGT), 70˚C (ACE), 55˚C (GNB3).

Figure 4

Evaluation of 7-deaza-dGTP for amplification of targets with greater than 70% GC content



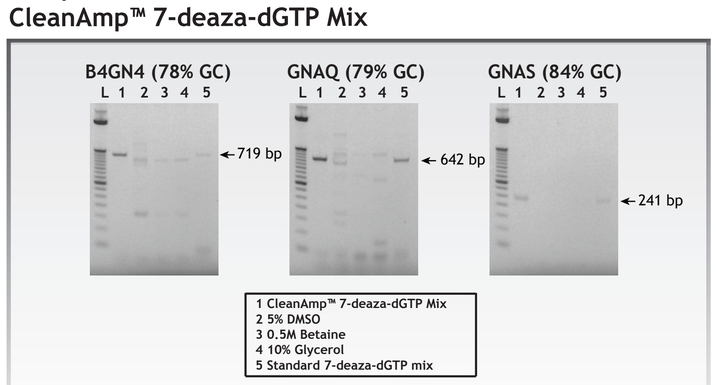
CleanAmp™ 7-deaza-dGTP Mix outperforms standard 7-deaza-dGTP in amplicon yield and specificity for targets with higher GC content.

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$); Standard dNTPs = 0.2 mM d(A, C, G,T)TP; Reactions containing 7-deaza-dGTP contained 0.2 mM d(A, C, T,)TP (standard or CleanAmp™); 0.05 mM dGTP (standard or CleanAmp™) and 0.15 mM 7-deaza-dGTP (standard or CleanAmp™); Primer conc.: 0.2 µM (B4GN4, DACT3, GNAQ), 0.1 µM (BRAF); 1.0 U *Taq* DNA polymerase; 0.2 ng/µL Human gDNA; 25 µL.

Thermal cycling conditions: 95˚C (10 min) [95˚C (40 sec), X˚C (1 sec), 72˚C (1 min)] 35X, 72˚C (7 min) (X= 67˚C (BRAF), 57˚C (B4GN4), 70˚C (DACT3), 66˚C (GNAQ - subjected to 40 thermal cycles).

Figure 5

Comparison of common GC-rich additives to the CleanAmp™ 7-deaza-dGTP Mix

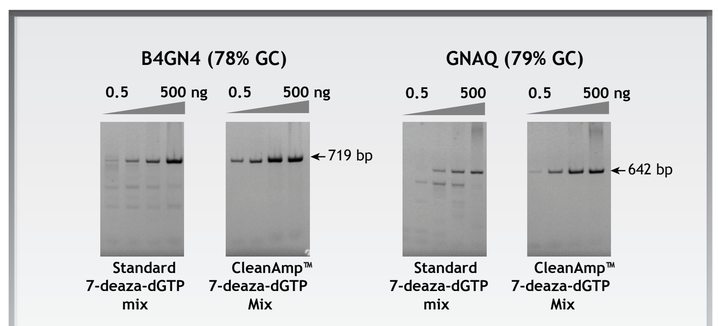


CleanAmp™ 7-deaza-dGTP Mix improves amplicon yield compared to standard 7-deaza-dGTP mix and outperforms other common GC-rich additives.

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$); Standard dNTPs = 0.2 mM d(A, C, G,T)TP; Reactions containing 7-deaza-dGTP contained 0.2 mM d(A, C, T,)TP (standard or CleanAmp™); 0.05 mM dGTP (standard or CleanAmp™) and 0.15 mM 7-deaza-dGTP (standard or CleanAmp™); Additives: 5% DMSO, 0.5 M Betaine, or 10% Glycerol; Primer conc.: 0.2 µM (B4GN4, GNAQ, GNAS); 1.0 U *Taq* DNA polymerase; 0.2 ng/µL Human gDNA; 50 µL.

Figure 6

Amplification of different input target concentrations using standard 7-deaza-dGTP mix and CleanAmp™ 7-deaza-dGTP Mix



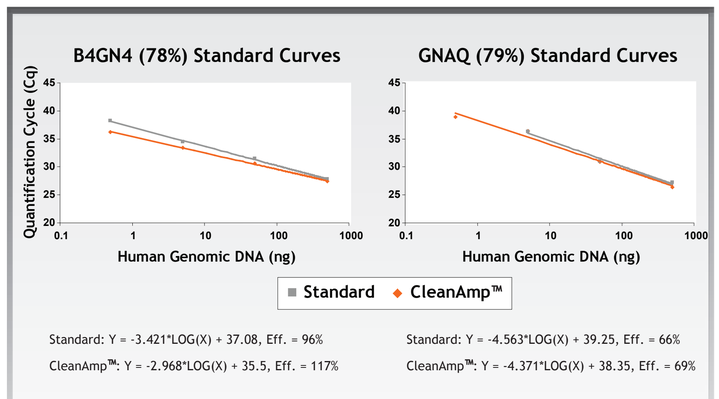
CleanAmp™ 7-deaza-dGTP Mix provides the greatest specificity and amplicon yield across a range of template concentrations.

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$), Primers (0.2 µM), 7-deaza-dGTP mix (standard or CleanAmp™) = 0.2 mM d(A, C, T,)TP; 0.05 mM dGTP, and 0.15 mM 7-deaza-dGTP, 1.0 U Taq DNA polymerase, 25 µL. Template: X ng Human gDNA.

Thermal cycling conditions: 95ºC (10 min); [95ºC (40 sec), X ºC (1 sec), 72ºC (60 sec)] 35X; 72ºC (7 min). X = 57˚C (B4GN4), 66˚C (GNAQ - subjected to 40 thermal cycles).

Figure 7

Analysis of Real-time PCR performance with standard 7-deaza-dGTP mix and CleanAmp™ 7-deaza-dGTP Mix



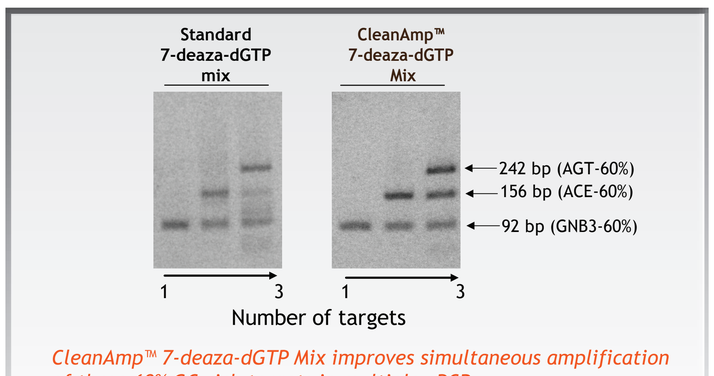
CleanAmp™ 7-deaza dGTP Mix allows for increased limit of detection and more efficient PCR reactions.

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$), Primers (0.2 µM), 7-deaza-dGTP mix (standard or CleanAmp™) = 0.2 mM d(A, C, T,)TP; 0.05 mM dGTP, and 0.15 mM 7-deaza-dGTP, 1.0 U *Taq* DNA polymerase, 0.1µM hydrolysis probe, 25 µL. Template: X ng Human gDNA.

Thermal cycling conditions: 95ºC (10 min); [95ºC (40 sec), X ºC (1 sec), 72ºC (60 sec)] 40X; 72ºC (7 min). X = 57˚C (B4GN4), 66˚C (GNAQ)

Figure 8

Comparison of 7-deaza-dGTP Mix (standard and CleanAmp™) in multiplex PCR

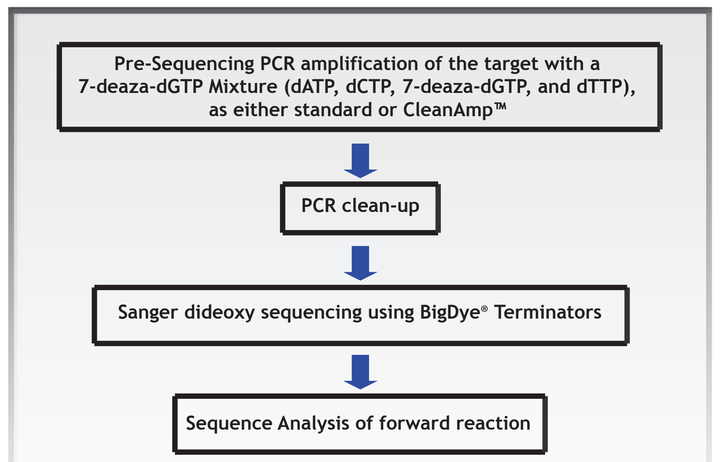


CleanAmp™ 7-deaza-dGTP Mix improves simultaneous amplification of three 60% GC-rich targets in multiplex PCR

PCR Conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$), 0.1U/µL *Taq* DNA Polymerase; 0.2 µM primers; Additional 60 mM KCl, 7-deaza-dGTP mix (standard or CleanAmp™) = 0.4 mM d(A, C, T,)TP; 0.10 mM dGTP, and 0.30 mM 7-deaza-dGTP; 0.02 ng/µL of Human genomic DNA; 25 µL.
Thermal Cycling Conditions:
95ºC 10min [95ºC for 40sec, 55 ºC for 1 sec, 72º for 1.08 min] x40, 72ºC @ 10 min.

Figure 9

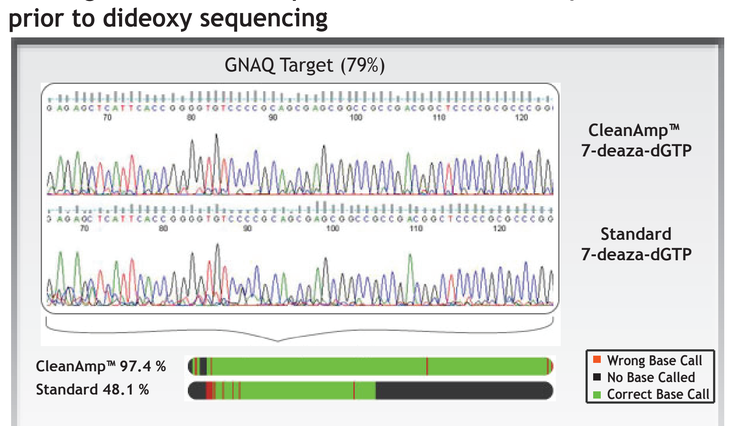
Approach to improving dideoxy sequencing results by use of 7-deaza-dGTP in the pre-sequencing PCR step



A complete substitution of 7-deaza-dGTP for dGTP in a pre-sequencing PCR reaction improves the quality of sequencing data

Figure 10

Investigation of CleanAmp™ 7-deaza-dGTP for amplification prior to dideoxy sequencing



The use of a CleanAmp™ version of 7-deaza-dGTP in a pre-sequencing PCR mix improves the quality of base calling and length of reads in sequencing reactions

Preamplification PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$); 0.2 mM d(A, C, T,)TP (standard or CleanAmp™) and 0.2 mM 7-deaza-dGTP (standard or CleanAmp™); [Complete substitution of 7-deaza-dGTP for standard dGTP]; Primer conc.: 0.2 µM GNAQ; 1.0 U *Taq* DNA polymerase; 0.2 ng/µL Human gDNA; 25 µL
Thermal cycling conditions: 95˚C (10 min) [95˚C (40 sec), 66˚C (1 sec), 72˚C (1 min)] 40X, 72˚C (7 min)
PCR Cleanup of 4 replicates combined
Sanger dideoxy sequencing using ABI Big Dye Terminators (Forward Reactions only)- Eton Biosciences.

Conclusion

1) CleanAmp™ 7-deaza-dGTP Mix allows for clean amplification of high GC-rich targets of up to 84% GC content by reducing off-target amplification and increasing amplicon yield.

2) CleanAmp™ 7-deaza-dGTP Mix outperforms other GC-rich amplification solutions.

3) CleanAmp™ 7-deaza-dGTP Mix improves amplification efficiency and limit of detection of targets with high GC content while mantaining a clean and robust product.

4) CleanAmp™ 7-deaza-dGTP Mix is able to successfully amplify three GC-rich targets in multiplex PCR.

5) A pre-sequencing version of the CleanAmp™ 7-deaza-dGTP Mix improves the quality of sequencing data for targets with high GC content by reducing background and improving base-calling.

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