CleanAmp™ Hot-Start 7-deaza-dGTP for Improved GC-rich PCR Amplification

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Abstract

PCR amplification of nucleic acids is a fundamental technique used in many molecular biology laboratories. Despite its widespread use, GC-rich regions of DNA sequence remain a challenge for amplification. Sequences high in GC content can form strong secondary structure, which prevents strand denaturation and blocks processive DNA polymerase amplification. As a consequence, mis-priming is prominent and can complicate specific product formation. Especially as applied to the molecular diagnosis of inheritable diseases, several assay modifications have been developed to improve the specificity of target amplification. These 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. Here, we show how 7-deaza-dGTP, a commonly used molecule to amplify GC-rich targets, can greatly 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. This Hot Start version of 7-deaza-dGTP, CleanAmp™ 7-deaza-dGTP improves the amplification of targets containing up to 80% GC content. Results were further improved when a Hot Start version of all dNTPs was employed, allowing for challenging targets of more than 85% GC content, such as Fragile X, to be amplified. Another benefit of this technology is in downstream sequencing reactions. PCR amplification of problematic targets with a Hot Start 7-deaza-dGTP mix prior to Sanger dideoxy sequencing can significantly improve the read quality along the entire sequence. In summary, the use of CleanAmp™ dNTPs simplifies GC-rich amplification and provides a valuable solution that can improve disease diagnosis.

Figure 1: CleanAmp™ Hot Start dNTP Chemical Structure

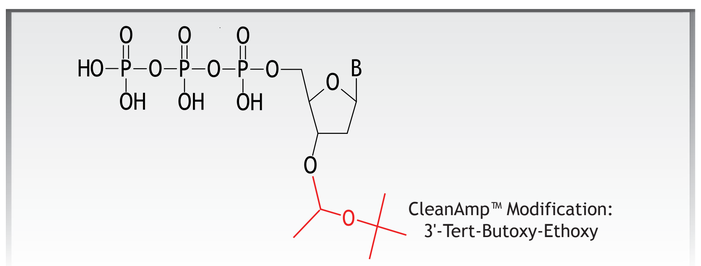
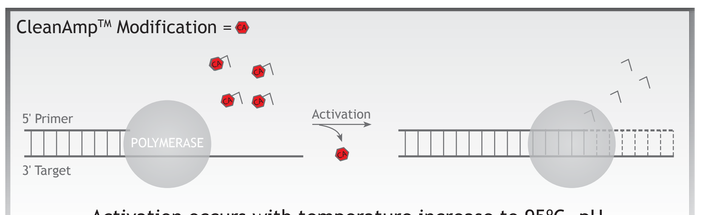


Figure 2: CleanAmp™ dNTP Activation Mechanism



Activation occurs with temperature increase to 95ºC, pH change or addition of chemical reagent.

Figure 3: 7-deaza-dGTP Reduces Secondary Structure Formation

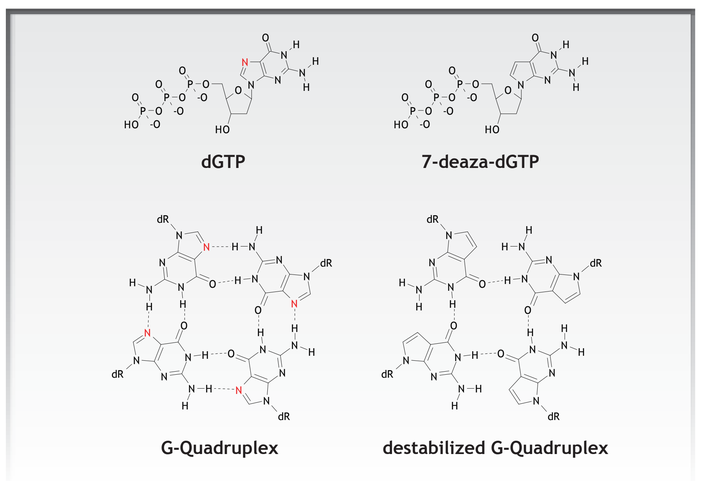
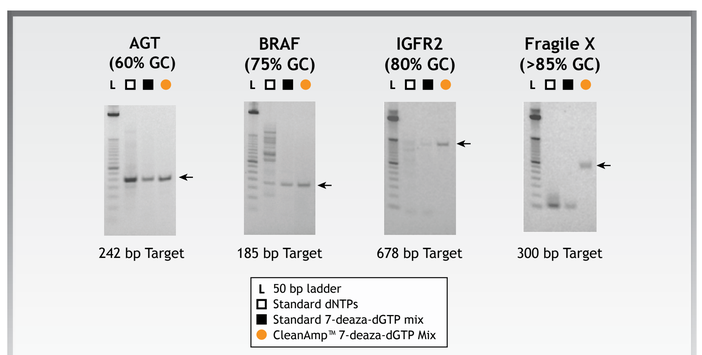
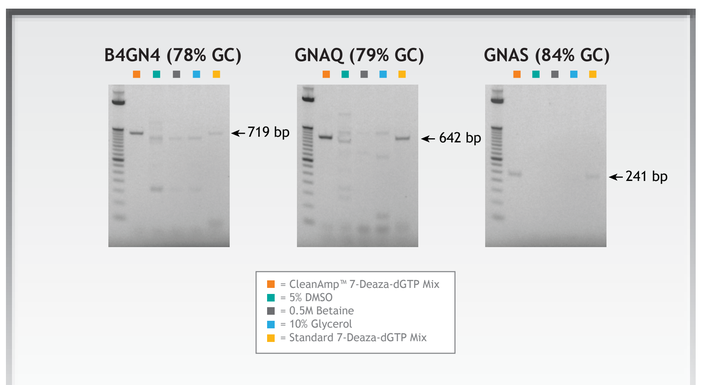


Figure 4: Improved Amplification Yield and Specificity for Targets Up to 85% GC-Content



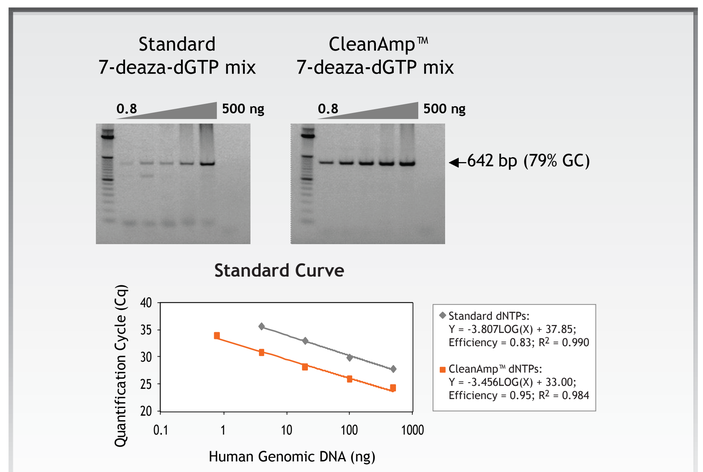
PCR conditions: PCR buffer, 7-deaza-dGTP Mix (standard[1] or CleanAmp™[2]), each at 1X or 1.5X), Primers (0.2 µM), DNA polymerase (1-1.25 U), human gDNA (5 ng), 25 µL.
Thermal cycling conditions: 98˚C(10 min); 35-40X [95˚C (40 sec), x˚C (1 sec), 72˚C (1 min)]; 72˚(7 min) where *x* is 61˚C (AGT), 67˚C (BRAF), 63˚C (IGFR2) or 65˚C (Fragile X).

Figure 5: CleanAmp™ 7-deaza-dGTP Outperforms Common Additives for GC-Rich Target Amplification



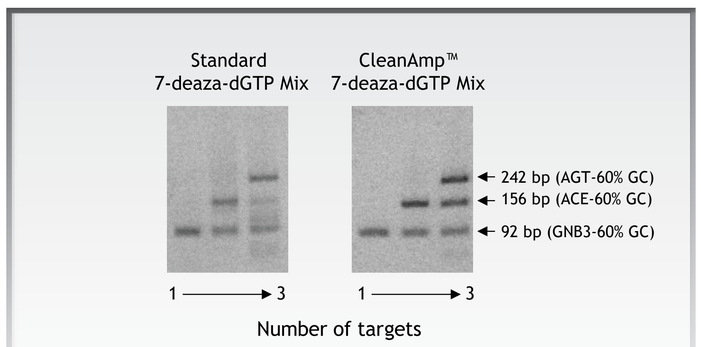
PCR conditions: PCR buffer, 7-deaza-dGTP Mix (standard[1] or CleanAmp™[2]), Additives, Primers (0.2 µM), *Taq* DNA polymerase (2.0 U), Human gDNA (10 ng), 50 µL.
Thermal cycling conditions: 95˚C(10 min); [95˚C (40 sec), x˚C (1 sec), 72˚C (1 min)] 35-40X; 72˚(7 min) where *x* is 57˚C (B4GN4), 66˚C (GNAQ) or 64˚C (GNAS).

Figure 6: Increased Specificity and Amplicon Yield Across a Range of Template Concentrations



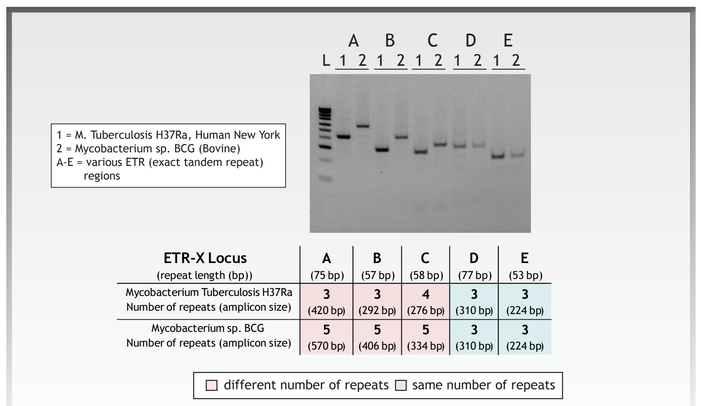
PCR conditions: PCR buffer, 7-deaza-dGTP Mix (standard[1] or CleanAmp™[2]), Primers (0.2 µM), hydrolysis probe (0.1 µM), *Taq* DNA polymerase (1.25 U), Human gDNA (0.8-500 ng), 25 µL.
Thermal cycling conditions: 95˚C(10 min); [95˚C (40 sec), 66˚C (1 sec), 72˚C (1 min)] 40X; 72˚(10 min).

Figure 7: Successful Multiplex PCR of GC-Rich Targets



PCR conditions: PCR buffer with 60 mM additional KCl, 7-deaza-dGTP Mix (standard[1] or CleanAmp™[2], each at 2X), Primers (0.2 µM), *Taq* DNA polymerase (2.5 U), Human gDNA (5 ng), 25 µL.
Thermal cycling conditions: 95˚C(10 min); [95˚C (40 sec), 55˚C (1 sec), 72˚C (1 min)] 40X; 72˚(10 min).

Figure 8: Bacterial Identification by Amplification of Variable Number of Tandem Repeats (VNTRs)

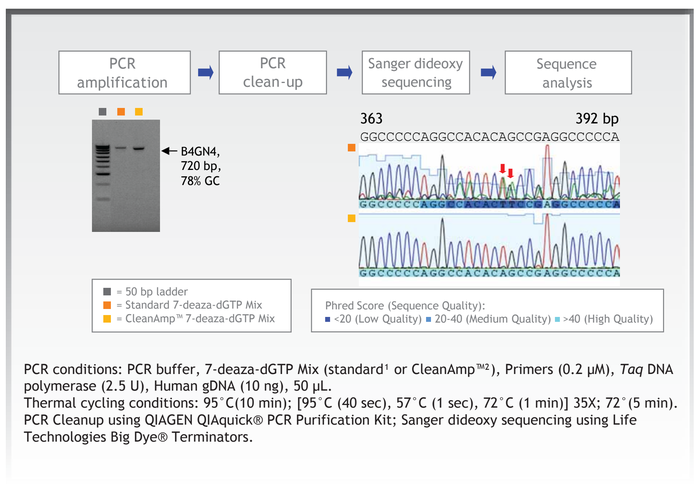


ETR-X Locus (repeat length (bp))	A (75 bp)	B (57 bp)	C (58 bp)	D (77 bp)	E (53 bp)
Mycobacterium Tuberculosis H37Ra Number of repeats (amplicon size)	3 (420 bp)	3 (292 bp)	4 (276 bp)	3 (310 bp)	3 (224 bp)
Mycobacterium sp. BCG Number of repeats (amplicon size)	5 (570 bp)	5 (406 bp)	5 (334 bp)	3 (310 bp)	3 (224 bp)

different number of repeats | same number of repeats

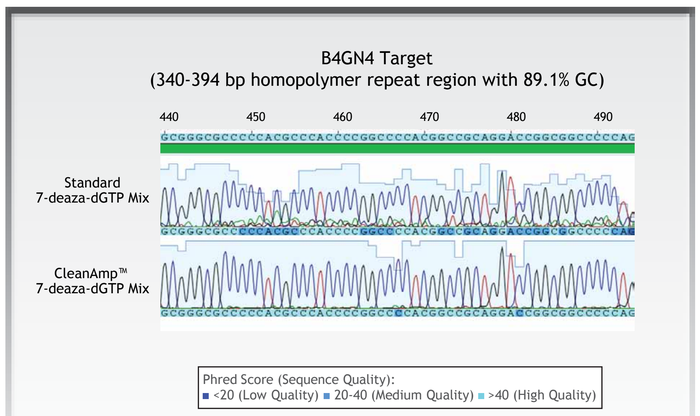
PCR conditions: CleanAmp™ GC-Rich Master Mix, Primers (0.2 µM), human or bovine BCG DNA (5 ng), 25 µL.
Thermal cycling conditions: 95˚C(10 min); [95˚C (40 sec), 60 or 65˚C (1 sec), 72˚C (1 min)] 35X; 72˚(7 min).

Figure 9: PCR Amplification of GC-Rich Targets Improves the Accuracy of Dideoxy Sequencing



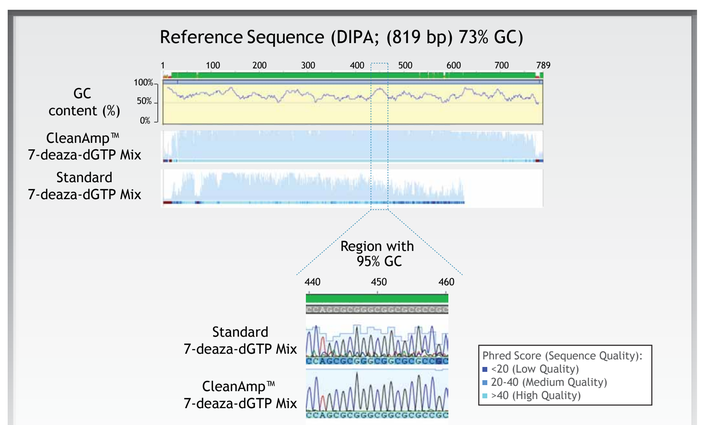
PCR conditions: PCR buffer, 7-deaza-dGTP Mix (standard[1] or CleanAmp™[2]), Primers (0.2 µM), *Taq* DNA polymerase (2.5 U), Human gDNA (10 ng), 50 µL.
Thermal cycling conditions: 95˚C(10 min); [95˚C (40 sec), 57˚C (1 sec), 72˚C (1 min)] 35X; 72˚(5 min).
PCR Cleanup using QIAGEN QIAquick® PCR Purification Kit; Sanger dideoxy sequencing using Life Technologies Big Dye® Terminators.

Figure 10: PCR Amplification Also Improves Sequencing Accuracy of Homopolymer Regions



PCR conditions: PCR buffer, 7-deaza-dGTP Mix (standard[1] or CleanAmp™[2]), Primers (0.2 µM), *Taq* DNA polymerase (2.5 U), Human gDNA (10 ng), 50 µL.
Thermal cycling conditions: 95˚C(10 min); [95˚C (40 sec), 57˚C (1 sec), 72˚C (1 min)] 35X; 72˚(5 min).
PCR Cleanup using QIAGEN QIAquick® PCR Purification Kit; Sanger dideoxy sequencing using Life Technologies Big Dye® Terminators.

Figure 11: PCR Amplification Allows for Longer Dideoxy Sequencing Results



PCR conditions: PCR buffer, 7-deaza-dGTP Mix (standard[1] or CleanAmp™[2]), Primers (0.2 µM), *Taq* DNA polymerase (2.5 U), Human gDNA (5 ng), 50 µL.
Thermal cycling conditions: 95˚C(10 min); [95˚C (40 sec), 57˚C (1 sec), 72˚C (1 min)] 35X; 72˚(5 min).
PCR Cleanup using QIAGEN QIAquick® PCR Purification Kit; Sanger dideoxy sequencing using Life Technologies Big Dye® Terminators.

Conclusion

- CleanAmp™ 7-deaza-dGTP Mix allows for clean amplification of high GC-rich targets of up to 85% GC content by reducing off-target amplification and increasing amplicon yield.
- CleanAmp™ 7-deaza-dGTP Mix outperforms other GC-rich amplification solutions.
- 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.
- CleanAmp™ 7-deaza-dGTP Mix is able to successfully amplify three GC-rich targets in multiplex PCR.
- A PCR step with CleanAmp™ 7-deaza-dGTP Mix prior to dideoxy sequencing improves the quality of sequencing data for targets with high GC content by reducing background and improving base-calling.

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LEGEND:

[1] 1X Standard 7-deaza-dGTP mix: 0.2 mM d(A, C, T,)TP; 0.05 mM dGTP and 0.15 mM 7-deaza-dGTP
[2] 1X CleanAmp™ 7-deaza-dGTP Mix: 0.2 mM CleanAmp™ d(A, C, T,)TP; 0.05 mM CleanAmp™ dGTP and 0.15 mM CleanAmp™ 7-deaza-dGTP

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