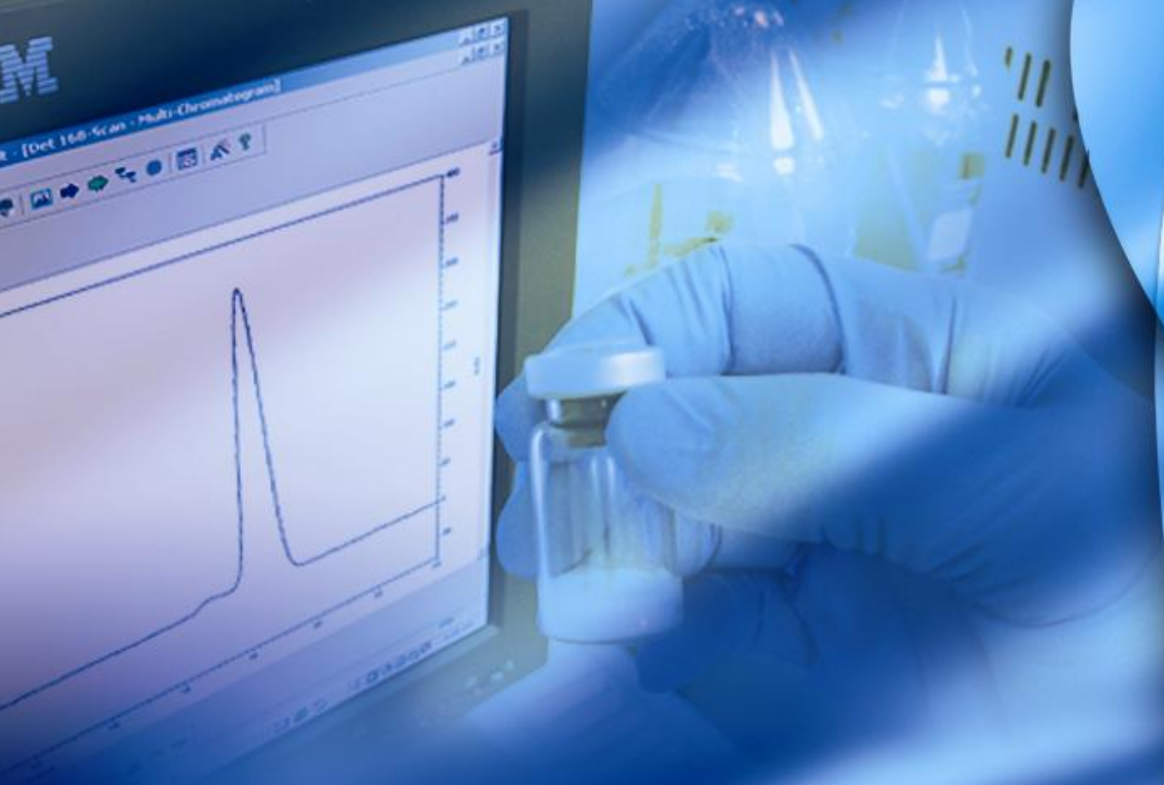

The Next Generation in Hot Start PCR - CleanAmp™ Primers and dNTPs

Natasha Paul, Ph.D.

TriLink BioTechnologies, Inc.

The Polymerase Chain Reaction

- Thermal cycling process that provides ~ 10^6-fold amplification of a nucleic acid target of interest

- Increasingly used in high-stringency applications, such as molecular diagnostics

- Plagued by competing off-target amplicon formation, such as mis-priming and primer dimer formation

- Hot Start activation: *A strategy to reduce non-specific amplification during the less stringent set-up stages of PCR*

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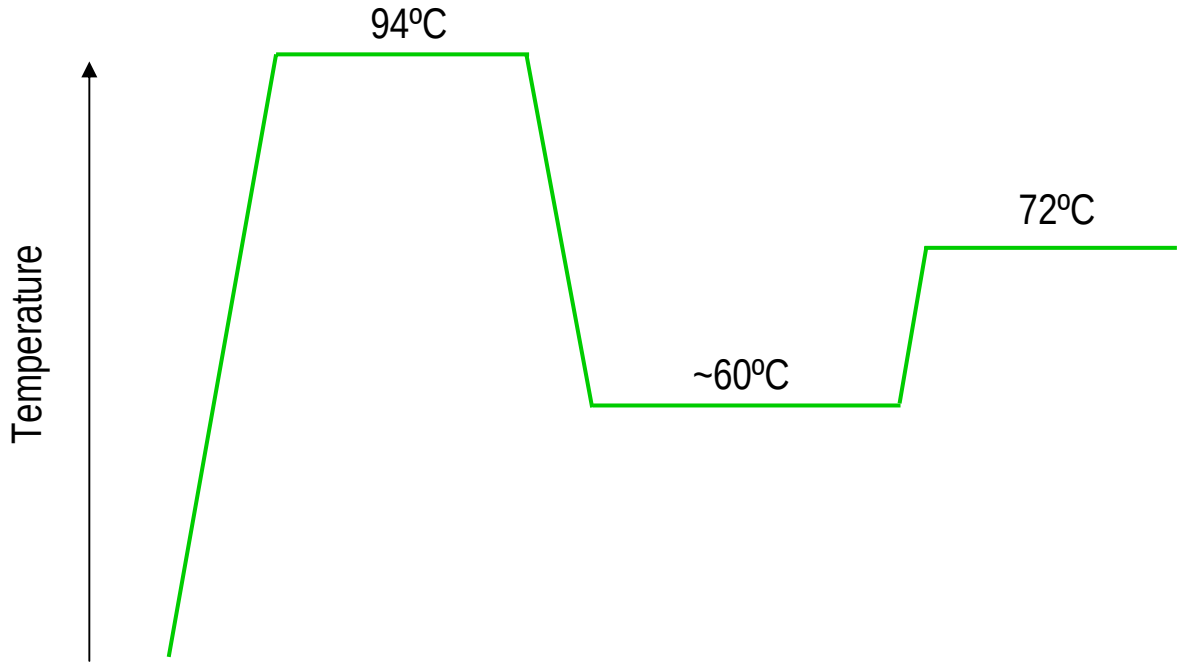
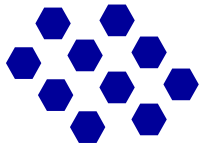
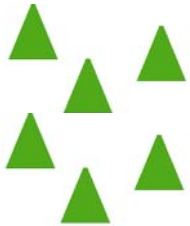
Hot Start Activation Approaches



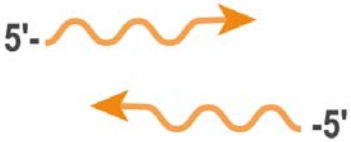


Magnesium ion

Thermostable DNA polymerase

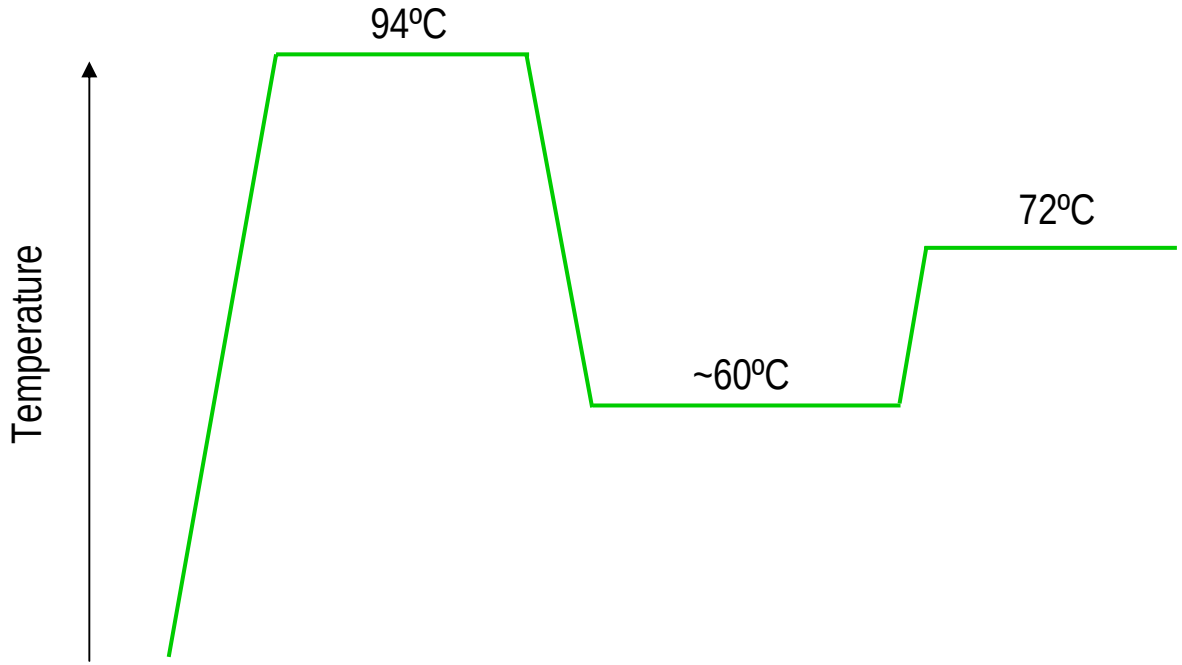


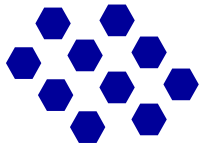
dNTPs



PCR primers

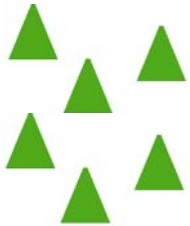
Hot Start Activation Approaches



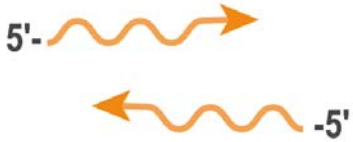


Magnesium ion

Thermostable DNA polymerase



dNTPs



PCR primers

Hot Start (CleanAmp™) Primers

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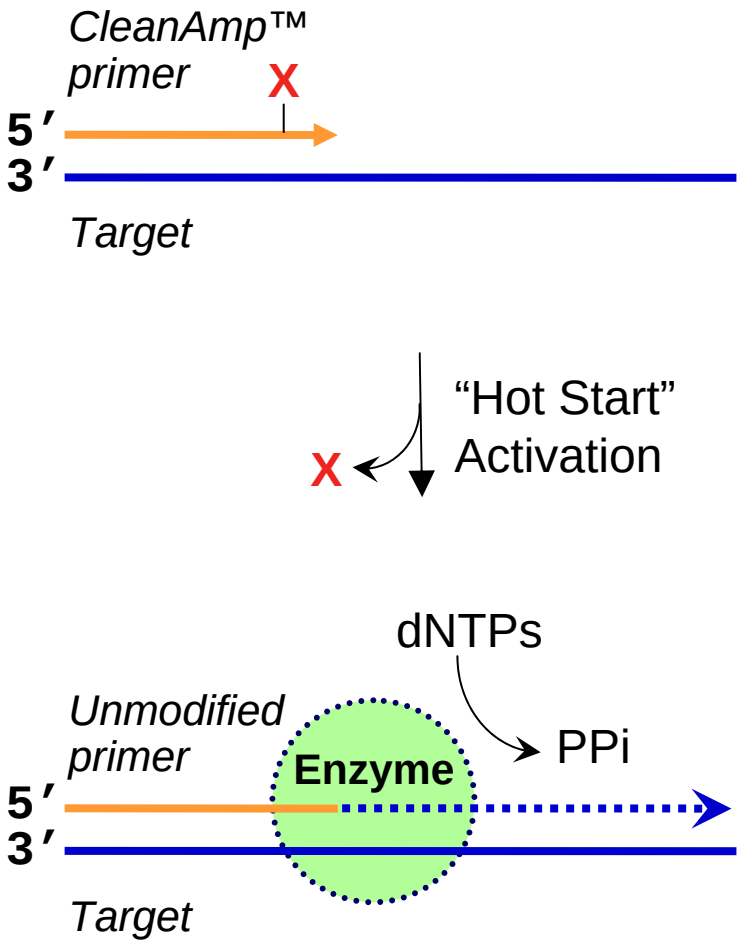
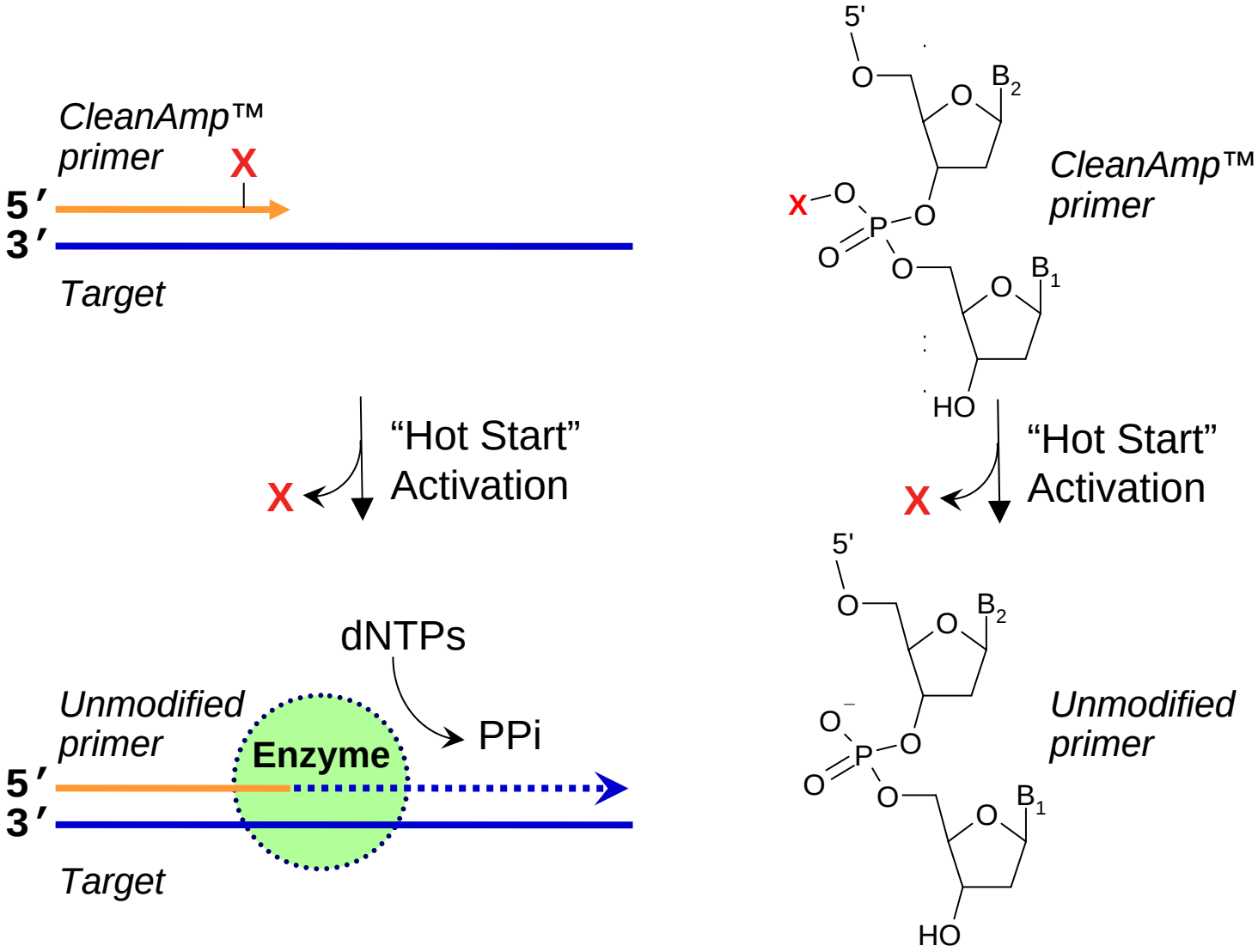
Proposed Mechanism of "Hot Start" (CleanAmp™) Primers



Proposed Mechanism of "Hot Start" (CleanAmp™) Primers



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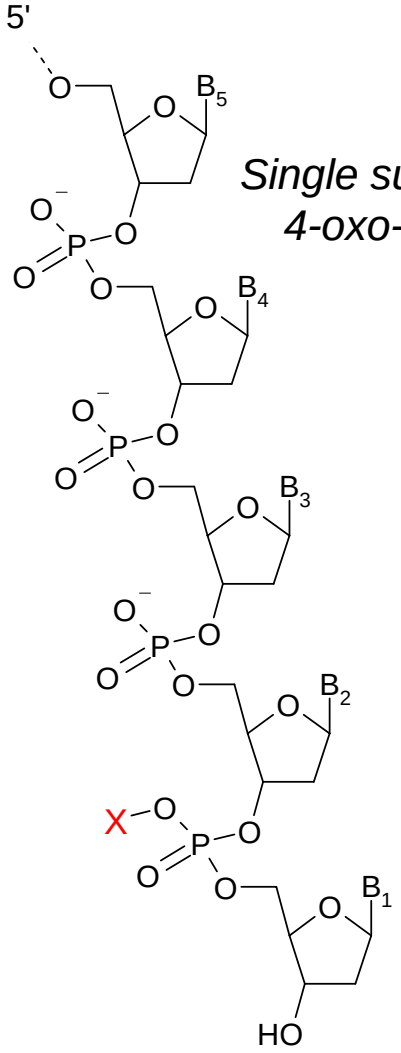
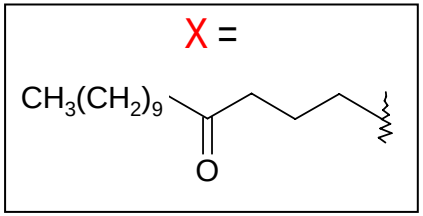
Thermolabile Phosphotriester Primer Protecting Group

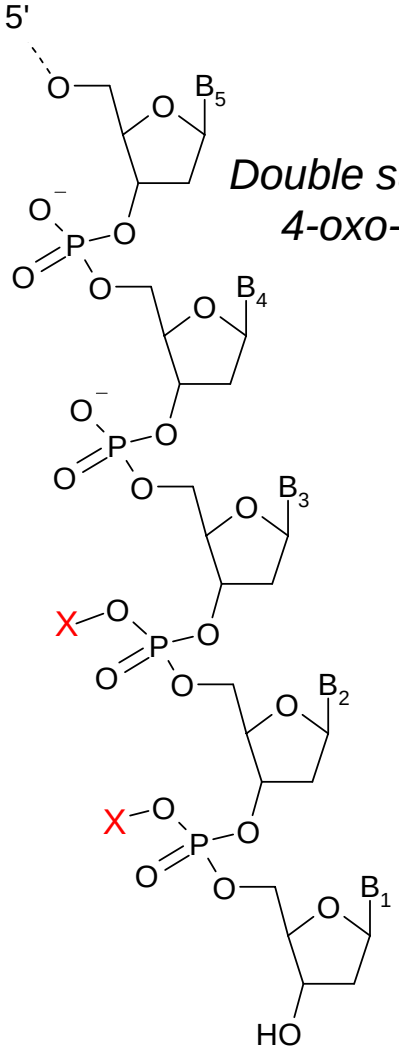
CleanAmp™ Turbo Primer



Single substitution of 4-oxo-tetradecyl



CleanAmp™ Precision Primer



Double substitution of 4-oxo-tetradecyl

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CleanAmp™ Primers – Application overview

CleanAmp™ Turbo Primers

- Fast cycling (2 step protocols)
- Multiplexed PCR (DNA template)

CleanAmp™ Precision Primers

- Standard cycling (3 step protocols)
- One-step reverse transcription PCR

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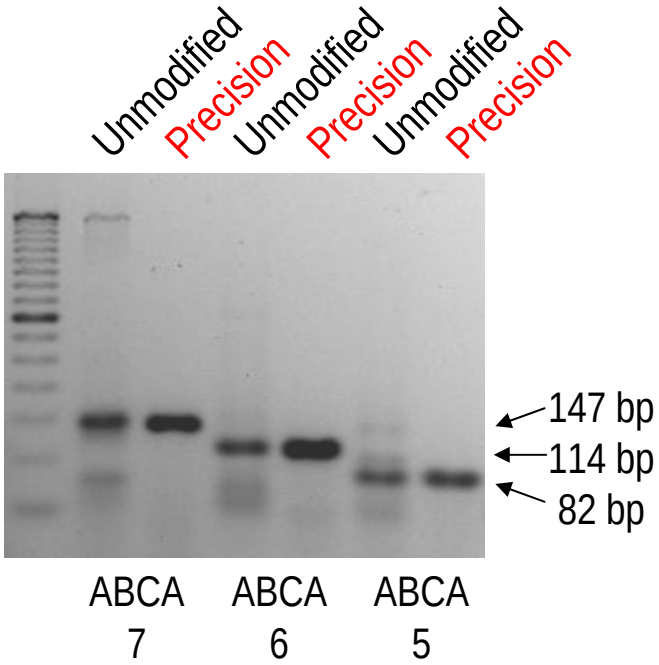
One-step reverse transcription PCR (RT-PCR) - CleanAmp™ Precision Primers



CleanAmp™ Precision PCR primer modifications improve one-step RT-PCR performance

Reaction conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$), gene-specific PCR primers (Unmodified or CleanAmp™ Precision) (0.5 μM), poly-dT_{18} primer (1 μM), 0.16 mM dNTPs, 0.5 μg of Human Trachea Total RNA, 5 U RNase Inhibitor, 50 U M-MLV RT, and 0.6 U *Taq* DNA Polymerase, 25 μL.
Thermal cycling conditions: 42°C for 30 min; 95°C for 10 min; 45 PCR cycles of [95°C for 30 sec, 60°C for 1 min]; 72°C for 5 min.

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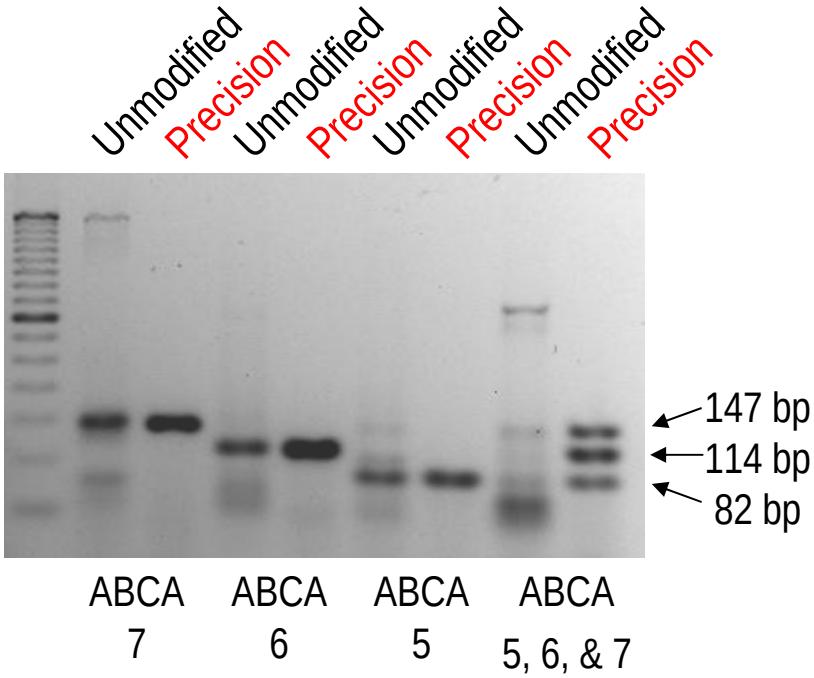
One-step reverse transcription PCR (RT-PCR) - CleanAmp™ Precision Primers



CleanAmp™ Precision PCR primer modifications improve multiplex one-step RT-PCR performance

Reaction conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$), gene-specific PCR primers (Unmodified or CleanAmp™ Precision) (0.5 μM), poly-dT_{18} primer (1 μM), 0.16 mM dNTPs, 0.5 μg of Human Trachea Total RNA, 5 U RNase Inhibitor, 50 U M-MLV RT, and 0.6 U *Taq* DNA Polymerase, 25 μL.
Thermal cycling conditions: 42°C for 30 min; 95°C for 10 min; 45 PCR cycles of [95°C for 30 sec, 60°C for 1 min]; 72°C for 5 min.

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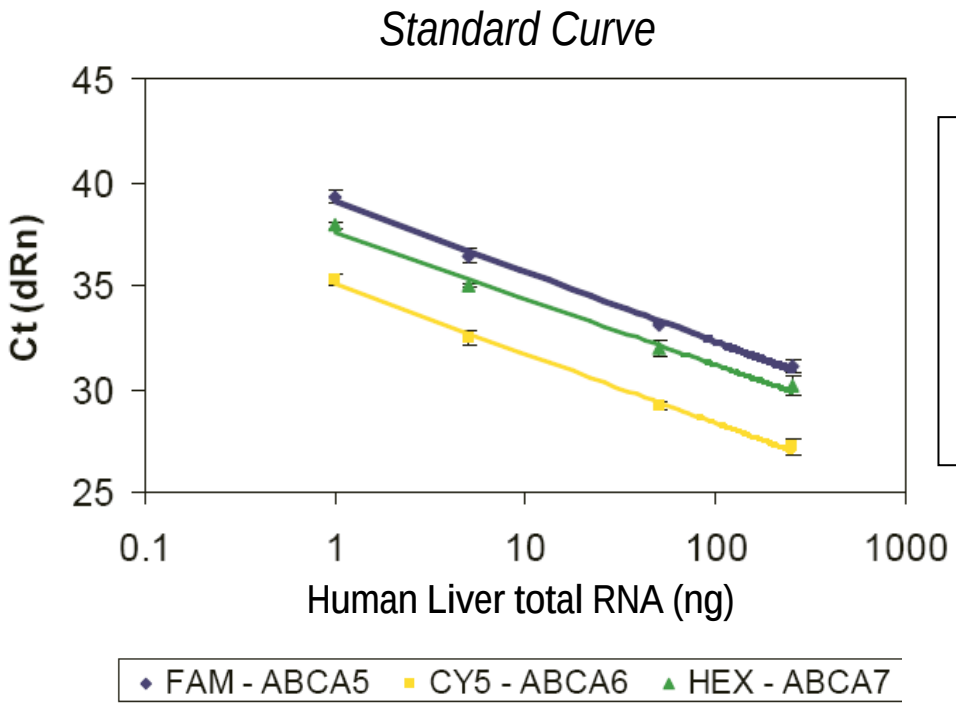
Real-time multiplexed one step reverse transcription PCR (RT-PCR)



ABCA5 : Eff. = 96.8%
Y = -3.402*LOG(X) + 39.08

ABCA6 : Eff. = 97.8%
Y = -3.377*LOG(X) + 35.07

ABCA7 : Eff. = 104.3%
Y = -3.224*LOG(X) + 37.58

CleanAmp™ Precision primer modifications show promise for relative RNA quantification in real-time one-step RT-PCR.

Reaction conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$), gene-specific PCR primers (CleanAmp™ Precision) (0.5 μM), poly-dT_{18} primer (1 μM), 0.16 mM dNTPs, 1-500 ng of Human Trachea Total RNA, 5 U RNase Inhibitor, 50 U M-MLV RT, and 0.6 U *Taq* DNA Polymerase, ROX and TaqMan® Probe (ABCA5 – FAM; ABCA6 - CY5; ABCA7 - HEX); 25 μL.
Thermal cycling conditions: 42°C for 30 min; 95°C for 10 min; 45 PCR cycles of [95°C for 30 sec, 60°C for 1 min]; 72°C for 5 min.

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CleanAmp™ Primers - Summary

- The use of thermolabile OXT phosphotriester modification groups provides a primer-based Hot Start approach to PCR

- CleanAmp™ Primer modifications provide improved PCR performance in a number of PCR-based applications

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Hot Start
(CleanAmp™)
dNTPs

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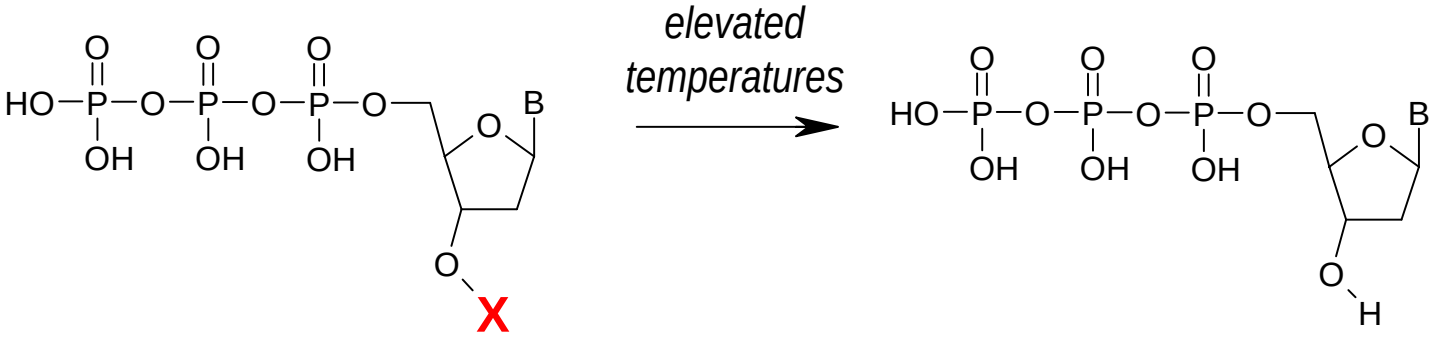
“Hot Start” activation of CleanAmp™dNTPs



X = thermolabile protecting group

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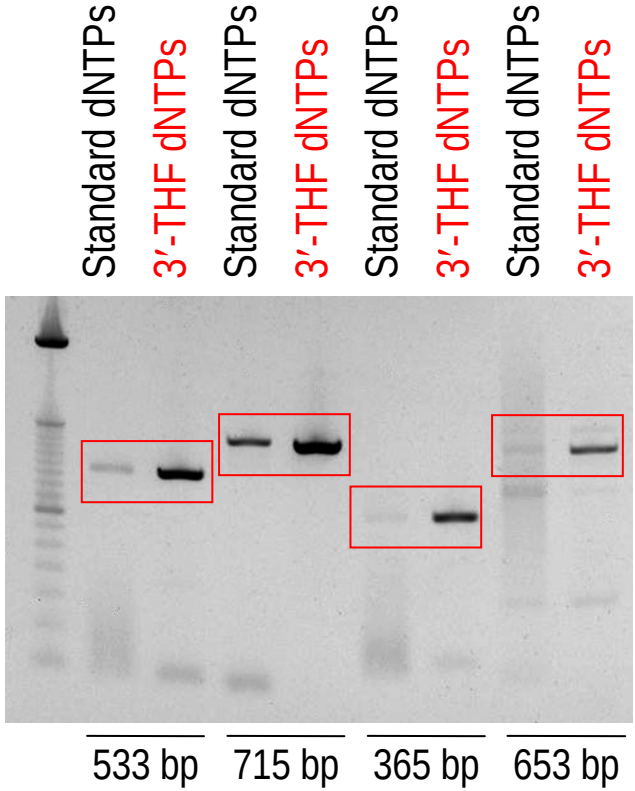
Performance of CleanAmp™ dNTPs – Multiple Targets



CleanAmp™ dNTPs improve PCR performance for different targets

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$), Primers (0.1-0.2 μM), 0.2 mM dNTPs, 1.25 U *Taq* DNA polymerase, 50 μL. **Template:** 5,000 copies Lambda gDNA (533 and 715 bp), 5 copies HIV-1 gDNA (365 bp), and 5ng Human gDNA (653 bp).
Thermal cycling conditions: **533 and 715 bp:** 95°C (10 min); [95°C (40 sec), 57°C (30 sec), 72°C (60 sec)] 35X; 72°C (7 min); **365 bp:** 94°C (10 min); [94°C (30 sec), 56°C (30 sec), 72°C (60 sec)] 35X; 72°C (7 min); **653 bp:** 94°C (2 min); [94°C (30 sec), 60°C (30 sec), 72°C (45 sec)] 35X; 72°C (7 min).

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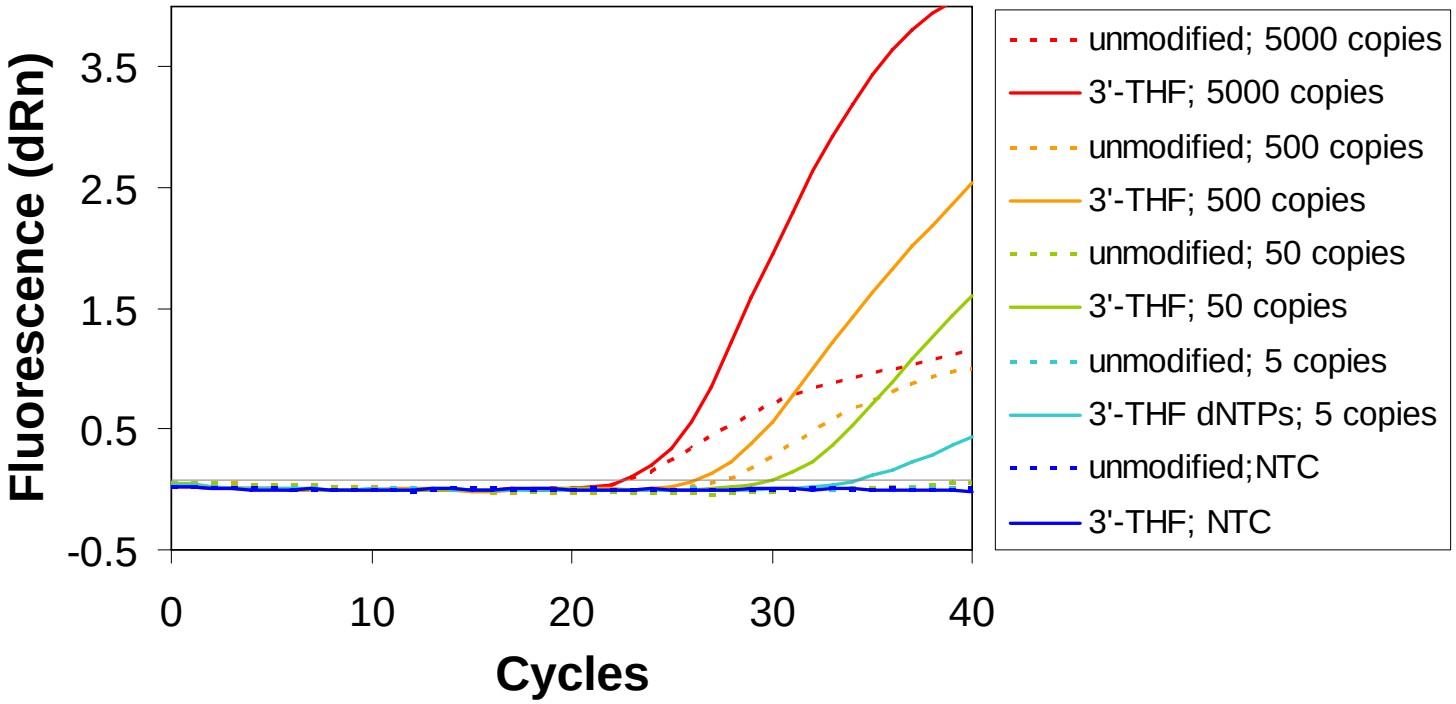
Real-time PCR performance of CleanAmp™ dNTPs

Amplification Plot



CleanAmp™ dNTPs allow for increased sensitivity in real-time PCR

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$), Primers (0.2 μM), 0.2 mM dNTPs, variable copies Lambda gDNA, TaqMan® probe (0.1 μM), ROX, 1.25 U Taq DNA polymerase, 50 μL.
Thermal cycling conditions: 95°C (10 min); [95°C (40 sec), 57°C (30 sec), 72°C (60 sec)] 40X.

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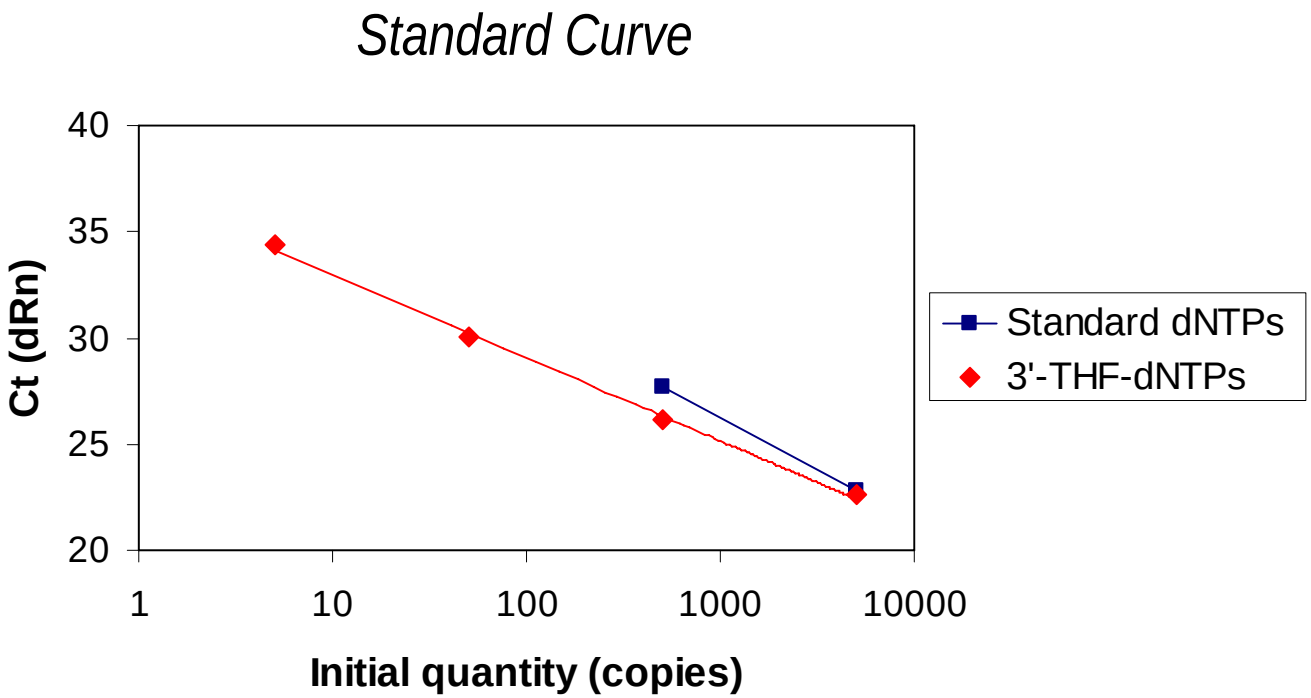
Real-time PCR performance of CleanAmp™ dNTPs



Standard dNTPs: Y = -4.902*LOG(X) + 40.94, Eff. = 60.0%
3′-THF-dNTPs: Y = -3.837*LOG(X) + 36.64, Eff. = 82.2%

CleanAmp™ dNTPs allow for increased limit of detection in real-time PCR

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$), Primers (0.2 μM), 0.2 mM dNTPs, variable copies Lambda gDNA, TaqMan® probe (0.1 μM), ROX, 1.25 U Taq DNA polymerase, 50 μL.
Thermal cycling conditions: 95°C (10 min); [95°C (40 sec), 57°C (30 sec), 72°C (60 sec)] 40X.

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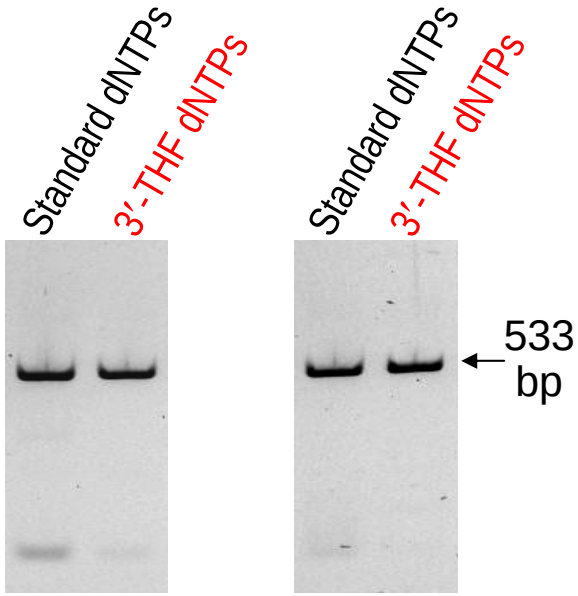
Evaluation of CleanAmp™ dNTPs for improved performance with Hot Start technologies



	CleanAmp™ Turbo Primers		CleanAmp™ Precision Primers	
Percent off-target:	10%	2%	2%	0%

The use of CleanAmp™ dNTPs with CleanAmp™ Primers enhances PCR specificity

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 40 mM KCl, 2.5 mM $MgCl_2$), Primers (0.2 µM) 0.2 mM dNTPs, 50 copies Lambda gDNA, 1.25 U *Taq* DNA polymerase, 50 µL. Thermal cycling conditions: 95°C (10min) [95°C (30 sec), 56°C (40 sec), 72°C (1 min)] 40X, 72°C (7min).

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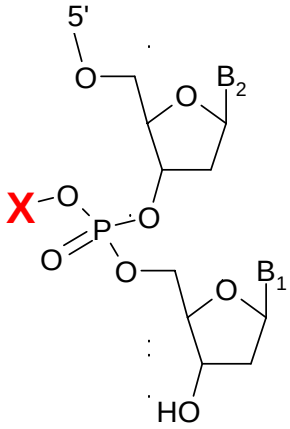
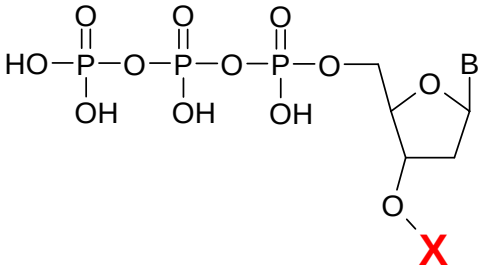
Overall summary





X = thermolabile protecting group

- *CleanAmp™ Primers and CleanAmp™ Primers dNTPs are two successful approaches to Hot Start activation in PCR*

- *CleanAmp™ Primers allow for temperature-dependent control of primer usage in PCR*

- *CleanAmp™ dNTPs show great potential as a more general Hot Start solution*

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Acknowledgements

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  - NIH awarding numbers (Primers): 1R43GM072177-01A1, 2R44GM072177-02, and 5R44GM072177-03
  - NIH awarding numbers (dNTPs): 1R43GM079836-01 and 2R44GM079836-02

- See http://www.trilinkbiotech.com/cleanamp/ for more information

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