Chemically Modified Primers for Improved Multiplexed PCR

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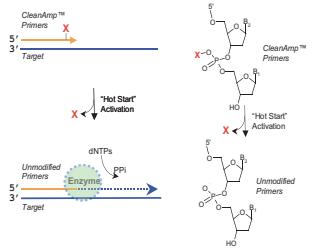
Abstract

Multiplex PCR is an advantageous technique used in PCR applications to amplify multiple targets in a single reaction. As useful as it is, this technique presents a new set of challenges that further complicates PCR setup. For example, reactions are more prone to off-target amplifications such as mis-priming and primer dimer due to the increased number of primer pairs. Furthermore, preferential amplification of certain targets leads to an unequal distribution of amplicon products, making quantification and detection of problematic targets extremely difficult. To improve upon the problems specific to multiplex PCR, we evaluated Hot Start modified primers which contain either one or two thermolabile 4-oxo-tetradecyl (OXT) modifications to prevent DNA polymerase extension at low-stringent temperatures, and that are released after a Hot Start activation step. Herein, we find that the singly-modified primers provide greater amplification efficiency, specificity, and yield in the multiplex amplification of DNA targets. In reverse transcriptase PCR (RT-PCR), the doubly-modified primers have been proven to be the optimal choice. The presence of two thermolabile protecting groups allows for an efficient one-step RT-PCR reaction that provides high specificity for multiple targets. TriLink's innovative technology represents a convenient tool for multiplex PCR amplification of DNA and RNA samples.

Figure 1

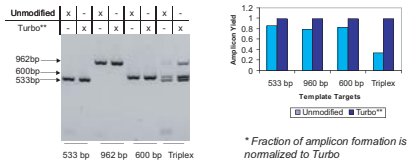
Proposed activation mechanism of CleanAmp™ Primers



X = Thermolabile protecting group

Figure 2

Utility of CleanAmp™ Turbo Primers in singleplex and multiplex PCR



*Fraction of amplicon formation is normalized to Turbo

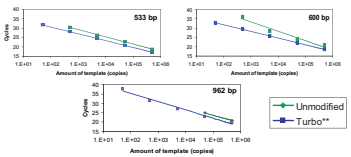
Turbo Primers demonstrate greatest amplicon yield in multiplex PCR relative to unmodified primers.

1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$), Primers (0.5 µM), 0.2 mM dNTPs, 50,000 copies Lambda gDNA, 1.25 U *Taq* DNA polymerase, 50 µL.

Thermal cycling conditions:
95°C (10 min); [95°C (40 sec), 56°C (30 sec), 72°C (2 min)]35X, 72°C (7 min)

Figure 3

Real-time analysis of multiplex PCR using CleanAmp™ Turbo Primers.



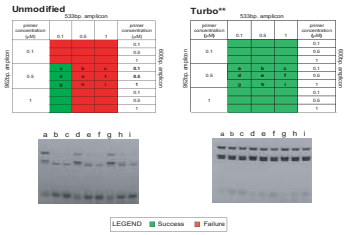
Turbo Primers provide increased sensitivity in Real-time PCR.

PCR conditions:
1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$), Turbo** CleanAmp™ Primers (0.2 µM), hydrolysis probe (0.1 µM), 0.2 mM dNTPs, 50 50,000 copies Lambda gDNA, 1.25 U *Taq* DNA polymerase, 25 µL; reactions performed in triplicate.

Thermal cycling conditions:
95°C (10 min); [95°C (40 sec), 56°C (30 sec), 72°C (2 min)]40X

Figure 4

Limited multiplex optimization with CleanAmp™ Turbo Primers



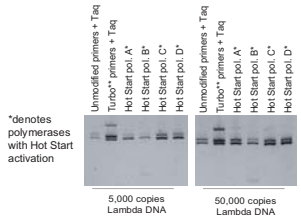
Turbo Primers effectively amplify all three targets over a wide range of primer concentrations.

PCR conditions:
1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$), Primers (0.5 µM), 0.2 mM dNTPs, 50,000 copies Lambda gDNA, 1.25 U *Taq* DNA polymerase, 50 µL.

Thermal cycling conditions: 95°C (10 min); [95°C (40 sec), 56°C (30 sec), 72°C (2 min)]35X, 72°C (7 min)

Figure 5

Hot Start technologies evaluation



Turbo Primers amplify all three targets efficiently compared to other Hot Start polymerases.

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$), Primers (0.075 µM), 0.2 mM dNTPs, 5 copies HIV-1 gDNA, DNA polymerases: 1.25 units of *Taq* and Hot Start DNA polymerases (A-D),

Thermal cycling conditions: 95°C (10 min); [95°C (40 sec), 56°C (30 sec), 72°C (1 min)] 35X, 72°C (7 min)

Figure 6

Increasing multiplex amplification using CleanAmp™ Turbo Primers

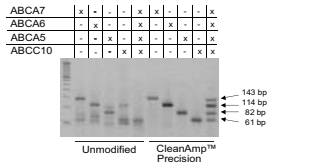
Turbo Primers can efficiently amplify at least nine targets ranging from 139 to 962 bp.

1X PCR buffer (20 mM Tris (pH 8.4), 90 mM KCl, 2.5 mM $MgCl_2$), Primers (0.2 µM), 0.4 mM dNTPs, 5,000 copies Lambda gDNA, 2.5 U *Taq* DNA polymerase, 50 µL.

Thermal cycling conditions:
95°C (10 min); [95°C (40 sec), 56°C (30 sec), 72°C (2 min)]35X, 72°C (7 min)

Figure 7

Singleplex and fourplex One-Step RT-PCR amplification using CleanAmp™ Precision Primers

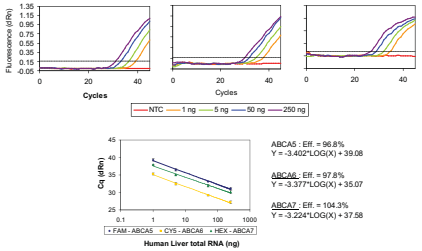


Precision Primers demonstrate greatest amplicon yield in multiplex One-Step RT-PCR relative to unmodified primers.

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl), 1.5 mM $MgCl_2$, Primers (0.5 µM), $polydT_{18}$ primer (1 uM), 0.16 mM dNTPs, 0.5 µg Human trachea total RNA, 50 U MMLV reverse transcriptase, 0.1 µM TM probe, 0.625 U Taq DNA polymerase, 50 µl.
Thermal cycling conditions:
42ºC (40 min); 95ºC (10 min); [95ºC (30 sec), 60ºC (30 sec), 72ºC (30 sec)] 30X; 72ºC (5 min).

Figure 8

Triplex One-Step RT-PCR amplification using CleanAmp™ Precision Primers

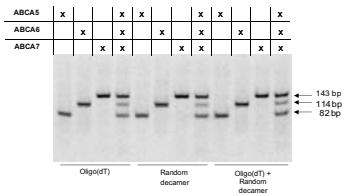


The use of Precision Primers in Real-time One-Step RT-PCR allows a wide range of linearity for input RNA.

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl), 1.5 mM $MgCl_2$, Primers (0.5 µM), $polydT_{18}$ primer (1 uM), 0.16 mM dNTPs, 1, 5, 10, 50, and 250 ng Human trachea total RNA, 50 U MMLV reverse transcriptase, 0.1 mM hydrolysis probe, 0.625 U Taq DNA polymerase, 50 µl.
Thermal cycling conditions:
42ºC (40 min); 95ºC (10 min); [95ºC (30 sec), 60ºC (30 sec), 72ºC (30 sec)] 30X; 72ºC (5 min).

Figure 9

Compatibility of CleanAmp™ Precision Primers with different RT priming approaches

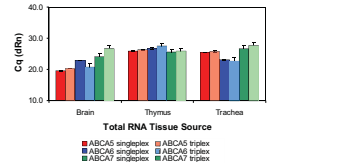


Precision Primers succesfully amplify three targets individually and simultaneously using different priming techniques.

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl), 1.5 mM $MgCl_2$, Primers (0.5 µM), $polydT_{18}$ primer (1 uM), 0.16 mM dNTPs, 0.5 µg Human trachea total RNA, 50 U MMLV reverse transcriptase, 0.1 µM hydrolysis probe, 0.625 U Taq DNA polymerase, 50 µl.
Thermal cycling conditions:
42ºC (40 min); 95ºC (10 min); [95ºC (30 sec), 60ºC (30 sec), 72ºC (30 sec)] 30X; 72ºC (5 min).

Figure 10

Triplex One-step RT-PCR amplification using CleanAmp™ Precision Primers



Precision Primers allow for reliable quantification of three targets individually and simultaneously in different tissues.

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl), 1.5 mM $MgCl_2$, Primers (0.5 µM), $polydT_{18}$ primer (1 uM), 0.16 mM dNTPs, 0.5 µg Human trachea total RNA, 50 U MMLV reverse transcriptase, 0.1 µM hydrolysis probe, 0.625 U Taq DNA polymerase, 50 µl.
Thermal cycling conditions:
42ºC (40 min); 95ºC (10 min); [95ºC (30 sec), 60ºC (30 sec), 72ºC (30 sec)] 30X; 72ºC (5 min).

Conclusion

1) CleanAmp™ Turbo Primers give optimal performance for multiplex amplification of DNA targets reducing optimization time.

2) Turbo Primers provide efficient amplification of up to nine targets ranging in size.

3) In multiplex Real-time PCR, Turbo Primers improve the limit of detection.

4) Precision Primers allow for both the RT and PCR steps of RT-PCR to be combined into a single reaction set-up without sacrificing specificity.

5) In one-step RT-PCR, Precision Primers can be used with different reverse transcription primers.

6) Triplex Real-time One-Step RT-PCR amplification in different tissues was possible with Precision Primers.

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** Denotes CleanAmp™ Primers containing 4-oxo-pentyl modifications

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