



CleanAmp™ Primers

The Next Generation in Hot Start PCR

Application Note



TriLink
BioTechnologies

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Introduction

The Polymerase Chain Reaction (PCR) is a commonplace molecular biological method to amplify a DNA target of interest. Although PCR is a powerful technique, its sensitivity and reproducibility are often plagued by off-target amplifications. These include primer dimer and mis-priming products caused by the hybridization of primers to one another or to non-specific regions on the template. Such off-target amplifications lower the efficiency of PCR by effectively sequestering PCR substrates, such as the primers, the DNA polymerase, and the dNTPs, from amplifying the desired template. At lower template concentrations, the problem of primer dimer formation and mis-priming is exacerbated as less template is available causing an increase in non-desired primer hybridization and extension.(1) Therefore, it is critical to substantially decrease or even eliminate off target amplification, especially when available template is limited.

Attempts have been made to alleviate the problem of primer dimer formation by the use of Hot Start technologies, which include physical separation of reaction components, inhibition of the DNA polymerase, and the use of accessory proteins. Many of the specialized DNA polymerase compositions can add significant cost to the reaction by the need for extensive manipulations, such as in the preparation of the DNA polymerase. In contrast, primers, which have CleanAmp™ thermolabile protecting groups, represent a simple approach to Hot Start activation in PCR. These modifications can be easily introduced to any primer sequence using standard solid phase oligonucleotide synthesis protocols. In addition, CleanAmp™ modifications are compatible with many commonly used DNA polymerases.

The introduction of thermolabile CleanAmp™ modifications into PCR primers allows for greater control of primer hybridization and extension during PCR. The thermolabile primer modifications prevent DNA polymerase extension at the lower, less-discriminating temperatures of reaction set-up and manipulation, but also display the flexibility to allow intended extension after activation at higher

temperatures (Figure 1). Furthermore, by using either the slow-releasing Precision Primers or the faster-releasing Turbo Primers, the rate of formation of unmodified primer can be controlled to suit your reaction needs. Herein, we investigate the utility of the CleanAmp™ Primer modifications in standard PCR protocols and in more advanced techniques such as multiplex PCR, one-step RT-PCR and fast cycling PCR.

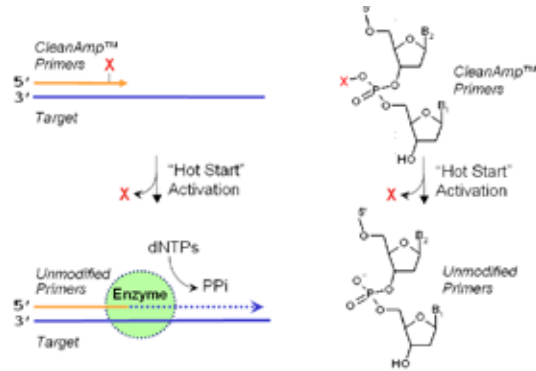


Figure 1: Hot Start Activation of CleanAmp™ Primers.

General Applications

CleanAmp™ Primers Significantly Lower, if not Eliminate Off-Target Amplification

Primer dimer formation has been found to be problematic in the amplification of a region of the HIV-1 tat genomic DNA.(1) In these studies, the reaction progression was monitored by removing aliquots after 30, 35 and 40 thermal cycles (Figure 2). Amplifications using unmodified PCR primers were found to be prone to robust primer dimer formation, which competes with the formation of the desired 365 bp amplicon (Figure 2A). By contrast, the introduction of Turbo Primers significantly reduced primer dimer formation and promoted

an even greater target yield as compared to the unmodified primers (Figure 2B). For Turbo Primers, only a slight amount of primer dimer is seen after 40 cycles. In the same system, the use of Precision Primers yielded only the desired amplicon, with no detectable primer dimer formation. However, while the slower release of the Precision protecting groups significantly reduced primer dimer formation, robust target amplification was slightly delayed at 30 thermal cycles, but fully recovered after 40 thermal cycles (Figure 2C). These studies demonstrated that Turbo Primers were able to efficiently form the desired amplicon, while significantly reducing primer dimer formation. Precision Primers were found to have the greatest utility when pure amplicon formation is required.

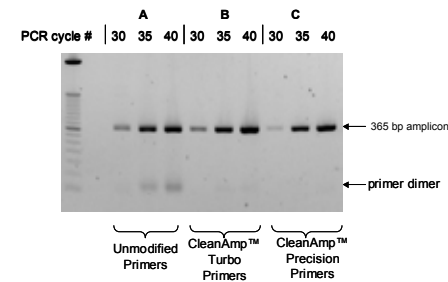


Figure 2: Endpoint PCR evaluation of CleanAmp™ Primers in a primer/template system prone to primer dimer. Aliquots of reaction were removed at 30, 35 and 40 cycles. PCR Conditions¹ on page 20.

Mis-priming can also be a significant hindrance to the fidelity and efficiency of amplification of the desired target. In comparison to unmodified primers, Turbo Primers reduce much of the mis-priming products, with Precision Primers providing the greatest benefit. This reduction in off-target amplification is evident over a wide range of input template concentrations with improved amplicon yield relative to unmodified primers.

Improve Specificity Over a Larger Range of Template Concentrations

Detection of a target at low concentrations is another difficulty encountered in PCR. Often, at low template concentrations, off-target amplifications compete with the desired amplification, complicating real-time PCR detection of the desired amplicon formation. CleanAmp™ Primers have been found to successfully amplify the correct amplicon at 10-100 fold lower template concentration as compared to unmodified primers. In Figure 3, the lower limit of detection of a 533 bp amplicon from Lambda genomic DNA was explored over a range of input template concentrations using SYBR Green® detection in real-time PCR. For the unmodified primers, the range of detection started above 500 copies. Because the amplification curve coincided with the no template control (NTC) curve at 500 copies or less, it would be impossible to differentiate between desired amplicon formation and other off-target formation.

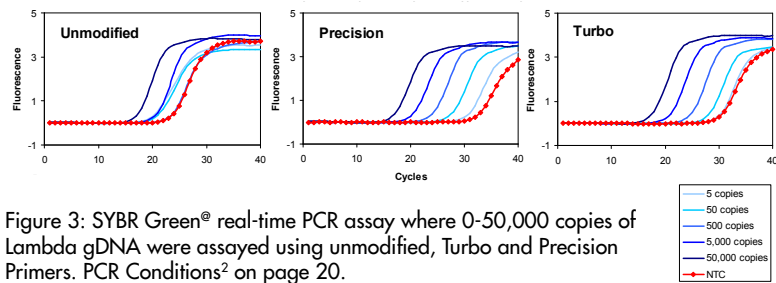


Figure 3: SYBR Green® real-time PCR assay where 0-50,000 copies of Lambda gDNA were assayed using unmodified, Turbo and Precision Primers. PCR Conditions² on page 20.

The use of Turbo Primers provides at least a ten-fold increase in detection, as the 50 copy concentration is distinguishable from the NTC curve. Precision Primers ultimately provide the greatest level of detection, detecting as low as five copies. This increased limit of detection using Precision Primers is indicative of their utility in a number of high-sensitivity downstream applications, such as single molecule detection.

CleanAmp™ Primers Outperform other Hot Start Technologies

Since CleanAmp™ Primers provide significant benefit relative to reactions with unmodified primers, experiments were then carried out to compare the performance of CleanAmp™ Primers to that of other Hot Start technologies. In these studies, the performance of unmodified primers with one of a series of Hot Start DNA polymerases, such as a chemically modified version of *Taq* (2), was compared to the performance of CleanAmp™ Primers with unmodified *Taq* DNA polymerase. Amplicon was formed with equal or much lower yield than reactions that employed unmodified *Taq* DNA polymerase with Precision Primers (Figure 4A, B). Moreover, Turbo Primers and unmodified *Taq* DNA polymerase gave the greatest benefit, as the amplicon yield was much higher than each of the Hot Start polymerases examined. These findings are significant, as they indicate that CleanAmp™ Primers and unmodified *Taq* DNA polymerase can be employed without compromising amplicon yield, while efficiently reducing primer dimer formation and mis-priming.

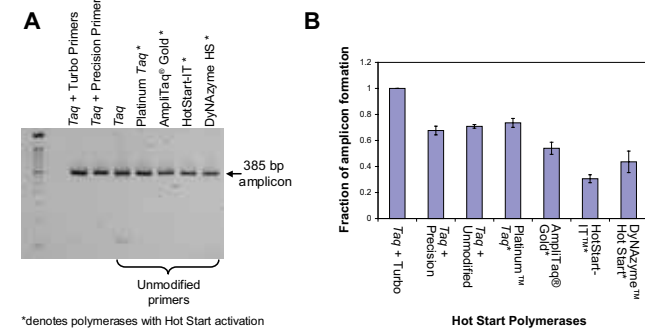


Figure 4: Comparison of CleanAmp™ Primers to other commercially available Hot Start DNA polymerases. A. Endpoint PCR analysis of amplification reactions containing five copies of HIV-1 genomic DNA. B. Graphical representation of the relative amplicon yield, normalized to reactions containing *Taq* DNA polymerase plus CleanAmp™ Turbo Primers.

CleanAmp™ Primers are Compatible with other DNA Polymerases

Taq DNA polymerase was used as a point of reference to determine whether Precision and Turbo CleanAmp™ Primers could be employed with other DNA polymerases in endpoint PCR experiments.

In the endpoint reactions, seven DNA polymerases devoid of Hot Start activation were evaluated for their ability to robustly form the desired 365 bp amplicon (Figure 5). Each of the DNA polymerases examined was able to support efficient amplification of the DNA target. In all cases, with the exception of Deep Vent™ and *Tfi* polymerase, the units of DNA polymerase were kept constant. Overall, CleanAmp™ Primers are versatile as they can be used with other thermostable DNA polymerases in endpoint PCR. This compatibility gives great potential for the implementation of CleanAmp™ Primers in a number of applications.

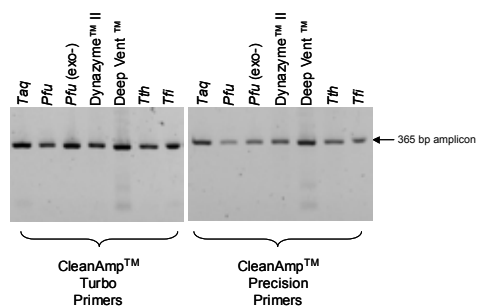


Figure 5: Evaluation of the performance of CleanAmp™ Primers in amplification reactions with a variety of thermostable DNA polymerases. PCR Conditions¹ on page 20.

Multiplex PCR Applications

One promising application of PCR is the ability to amplify multiple targets in a single reaction. This approach, known as multiplex PCR, employs a distinct primer pair for each amplicon of interest. This application has been an essential tool for many different medical, diagnostic and scientific applications, such as viral screens(3), where PCR based assays have proven to be more sensitive and less time consuming than traditional cell culture tests(4).

Although multiplex PCR has many advantages, there are inherent problems that inhibit robust amplification. One major factor is the increased propensity for primer dimer formation(5), which results from the larger number of unique primer sequences in the reaction. Another challenge of multiplex PCR is the preferential amplification of certain targets(6). Therefore the design of multiple primer pairs that are both specific for a target of interest and exhibit a low level of off-target amplicon formation can be a challenge. Coupled with this decreased flexibility in primer design, individual primer pair concentrations must be optimized, such that amplification efficiencies of all targets are similar(7).

This is a time consuming process, which has a low probability of success, should off-target amplicon formation dominate the reaction. Below, the ability of CleanAmp™ Primers to improve the specificity of amplicon formation for all targets in multiplex PCR is evaluated. The ability of CleanAmp™ Primers to reduce other competing off-target amplification, in single target reactions was applied to a multiplex PCR assay, where amplicon yield and PCR efficiency are extremely sensitive to primer dimer formation. Findings revealed minimal optimization of the design and concentration of the CleanAmp™ Primers with CleanAmp™ Turbo Primers improving with reduced primer dimer formation.

Multiplexed Target Amplification at Low Template Concentrations

When individual assays are combined into a single, multiplexed PCR assay, often template concentration must be increased to compensate for inefficient amplification. However, in clinical settings, where template sample is limited, increasing template concentration is not an option.

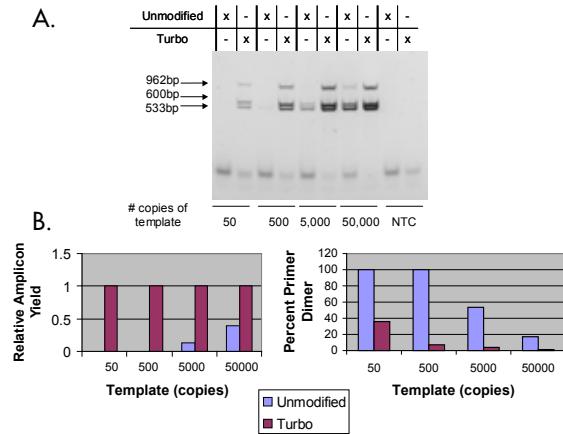


Figure 6: Multiplex reactions over a wide range of template amounts. A. Performance of Turbo and unmodified primers with a varying amount of template. B. Quantitative analysis of amplicon yield and primer dimer formation for experiment performed in Figure 6A. For each template concentration, amplicon yield was normalized to Turbo. PCR Conditions¹ on page 20.

In these cases where greater sensitivity is necessary, Turbo Primers have demonstrated much promise. When compared to unmodified primers, amplicon formation in a triplet reaction was detected at 100-fold lower input of template when using Turbo Primers (Figure 6A), with Turbo Primers efficiently detecting 50 copies of Lambda genomic DNA, where unmodified primers could only detect 5,000 copies. Furthermore, the longer targets appeared to amplify less efficiently than the shorter targets when using unmodified

primers, with the longest 962 bp amplicon not forming until 50,000 copies of template were employed. On the other hand, at all template concentrations examined, Turbo Primers amplified all three targets with similar efficiency. Additionally the use of CleanAmp™ Primers improved PCR performance by reducing primer dimer formation (Figure 6B). In summary, efficient amplification by Turbo Primers is less restricted by target size limitations, all three amplicons being formed over a broad range of input template concentrations at increased sensitivity.

CleanAmp™ Turbo Primers Outperform in Real-Time Multiplex PCR

To confirm that the range of detection was also reproducible in quantitative real-time PCR, a duplex reaction using Taqman® probe detection was performed. Much like the endpoint experiments, at low template concentrations, the detection of amplification is much more sensitive using CleanAmp™ Turbo Primers. In this duplex reaction, the difference in C_q between unmodified and Turbo Primers increased as template concentration decreased (Figure 7). In some cases, such as in the L600 target, no C_q is observed at 50 copies for the unmodified primers. Turbo Primers provide earlier detection of successful amplification, whereas amplicon detection with unmodified primers is delayed or not present.

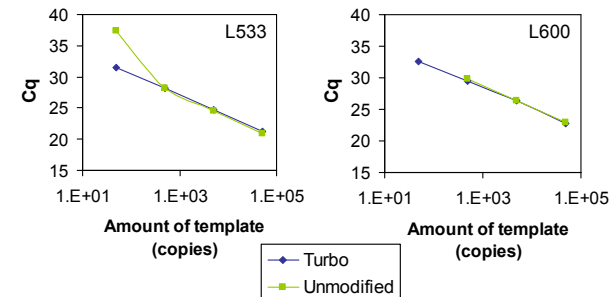


Figure 7: Real-time Multiplex PCR detection of a wide range of template amounts. Comparison of Turbo and unmodified primers varying concentration of template. PCR Conditions¹ on page 20.

Overall, the use of CleanAmp™ Turbo Primers in multiplex PCR provides several advantages, which include greater amplicon yield and lower primer dimer formation. Turbo Primers provide great flexibility in assay design, as a wide range of primer concentrations produce robust, non-preferential amplification. Furthermore greater sensitivity is achieved for both endpoint and real-time assays, with a 100-fold increase in the limit of detection.

One-step RT-PCR Applications

With the advent of microarrays, the need to validate the massive amount of gene expression results has grown significantly. Reverse transcription PCR (RT-PCR) has become the gold standard for validation of microarray gene expression profiles(8,9). The typical RT-PCR reaction consists of a two-step protocol that involves a lower temperature reverse transcription step followed by an elevated temperature PCR step(10). The extra manipulation procedures inherent to a two-step protocol can introduce opportunities for contamination. A one-step RT-PCR protocol provides a streamlined, high-throughput technique that reduces the chances of contamination(11). Another advantage for a one-step protocol is that replicates will repeat both the reverse transcription and the PCR step. However, one-step RT-PCR is not without its own inherent problems. In many cases one-step RT-PCR reactions are not as sensitive as two-step(12,13). The lack of sensitivity can be the result of reverse transcriptase(14) or DNA polymerase(15) mediated extension of primers to form primer-dimer and/or non-specific products at the less stringent temperatures of reverse transcription. To improve the sensitivity and specificity of RT-PCR, inhibition of such primer extension at lower temperatures is required.

One approach to improving the specificity of one-step RT-PCR is to employ CleanAmp™ Primers. By introducing a CleanAmp™ Primer pair, only the RT primer can elongate during reverse transcription. This reduces lower-temperature, non-specific amplicon formation from extension of PCR primers. At higher temperature, the

CleanAmp™ Primers are activated, allowing for greater specificity of primer extension during PCR. CleanAmp™ Primers provide a solution to non-specific amplifications and also enable other more universal RT priming methods for applications such as multiplex one-step RT-PCR.

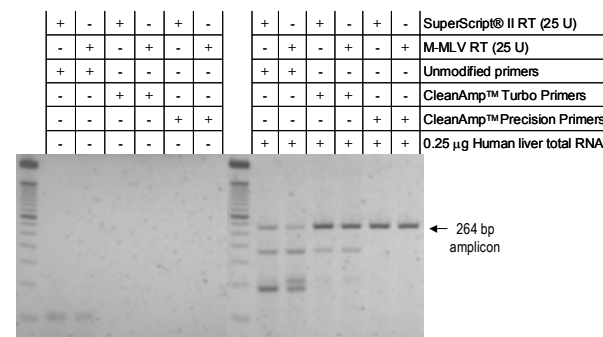


Figure 8: Evaluation of CleanAmp™ Primers in one-step reverse-transcription PCR using SuperScript® II Reverse Transcriptase (Invitrogen) and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen). For the gene of interest, the PCR primers were unmodified, contained CleanAmp™ Turbo modification or contained CleanAmp™ Precision modification. Reverse transcription utilized a polydT₁₈ primer. Reactions contained Taq DNA polymerase and SuperScript® II or M-MLV Reverse Transcriptase. PCR Conditions³ on page 20.

CleanAmp™ Primers Improve Sensitivity and Specificity Regardless of Reverse Transcriptase (RT) Enzyme

CleanAmp™ Primers decrease competing PCR primer extension during the RT step with a number of commonly used RT enzymes. To illustrate this, PCR primer sequences were prepared as either a) standard, unmodified primers or b) one of two types of CleanAmp™ Primers(16,17). CleanAmp™ Turbo Primers and slower-releasing CleanAmp™ Precision Primers differ in the rate of temperature-induced

formation of the corresponding unmodified primer. The following RT enzymes were tested: SuperScript® II Reverse Transcriptase (Invitrogen) and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen) (Figure 8), with similar results were found for both RT enzymes. The use of unmodified PCR primers resulted in formation of several non-specific amplicons, with the desired amplicon (264 bp) being formed at low relative abundance. Amplicon formation was enriched when CleanAmp™ Turbo Primers were used. In the case of CleanAmp™ Precision Primers, amplicon formation was also enriched to a slightly lesser degree. However, reactions containing Precision Primers displayed improved specificity.

CleanAmp™ Primers can also be utilized with reactions employing either total RNA or poly(A)+ RNA. Experiments have shown that with various RNA tissue sources, one-step RT-PCR displayed improved specificity when CleanAmp™ Primers were employed. Furthermore, one-step RT-PCR experiments have been evaluated in real-time qRT-PCR using CleanAmp™ Turbo and Precision Primers. The use of CleanAmp™ Primers enhanced the specificity of the reaction compared to the unmodified primers. CleanAmp™ Turbo Primers displayed the most significant increase in amplicon formation. Overall, CleanAmp™ Primers demonstrated marked improvement of sensitivity and specificity in one-step endpoint and real-time RT-PCR protocols.

CleanAmp™ Primers Improve Specificity of Duplex One-Step RT-PCR

CleanAmp™ Primers were examined for their ability to support multiplexed one-step RT-PCR. Gene A (264 bp) and Gene B (205 bp) were employed in singleplex and duplex reactions (Figure 9). In both singleplex and duplex reactions, unmodified primers produced off-target amplification products. CleanAmp™ Turbo and CleanAmp™ Precision Primers improved the specificity of singleplex reactions, CleanAmp™ Precision Primers providing the highest enrichment in multiplex amplification.

This unique thermolabile modification protects PCR primers from extension during the RT step until thermally activated in the PCR step, thereby reducing non-specific product formation in one-step RT-PCR protocols. Non-Hot Start DNA polymerases, such as *Taq* DNA polymerase, and standard reverse transcriptases, such as M-MLV reverse transcriptase, are recommended. There is no need to use more expensive modified DNA polymerases or RT enzymes. Furthermore, poly dT₁₈ or random primers can be employed during the cDNA synthesis steps instead of gene-specific primers. This universal priming method allows for a more streamlined approach to multiplex one-step RT-PCR reactions. This is also a key advantage of CleanAmp™ Primers because other one-step RT-PCR kits do not recommend the use of poly dT₁₈ or random primers. In summary, CleanAmp™ Precision Primers are a significant improvement over unmodified primers when used in one-step RT-PCR.

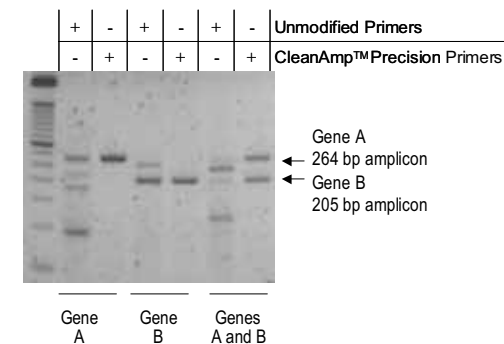


Figure 9: Evaluation of CleanAmp™ Primers in one-step reverse-transcription PCR in Human Liver Total RNA (Clontech) in singleplex and duplex. For the genes of interest (Gene A and Gene B), the PCR primers were unmodified or contained CleanAmp™ Precision modifications. Reverse transcription utilized a polydT₁₈ primer. Reactions contained *Taq* DNA polymerase and M-MLV Reverse Transcriptase. PCR Conditions⁴ on page 20.

Fast PCR Thermal Cycling Applications

In PCR applications such as diagnostics or medical testing, efforts have been directed at reducing the duration of the PCR amplification step, allowing for faster results and greater throughput. However, these faster cycling PCR protocols also have some drawbacks including a decrease in sensitivity and a loss in reproducibility(18). One suggested solution to decreased sensitivity is to increase primer concentration. The main complication encountered with this solution is that an increase in primer concentration often leads to higher off-target amplification, which can increase the false positive rate in real-time experiments, especially with SYBR Green® detection(18). Therefore, prevention of off-target amplification at high primer concentration could lead ultimately to an increase in sensitivity when using fast cycling PCR. Herein, the utility of CleanAmp™ Turbo Primers in preventing off-target amplification at high primer concentration using fast cycling PCR protocols will be demonstrated.

CleanAmp™ Turbo Primers Decrease Off-Target Amplification in Fast Cycling PCR

Turbo Primers were evaluated for their ability to reduce off-target amplification and to improve the sensitivity of real-time SYBR Green® assays using a typical fast cycling protocol (Figure 10). When unmodified primers were employed, interpretation of the amplification plots was complicated by strong amplification of the NTC sample. Although 125 copies of template DNA was accurately detected, the NTC sample amplified before each of the subsequent lower template concentrations. In comparison, the use of CleanAmp™ Turbo Primers allowed for detection down to a single copy. The NTC amplification curve did not cross the threshold line (C_q) during the 40 thermal cycles of the experiment. In addition, when the C_q values were plotted in standard curves (Figure 10B), CleanAmp™ Turbo Primers exhibited greater linearity compared to unmodified primers. This characteristic is most likely related to the

suppression of primer dimer formation when CleanAmp™ Turbo Primers are used and demonstrates Turbo Primer's increased specificity over a large range of template concentrations.

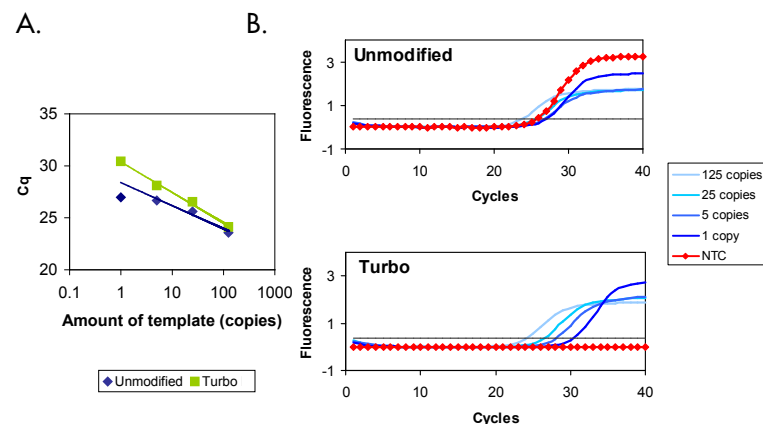


Figure 10: A. Amplification plots of CleanAmp™ Turbo Primers and unmodified primers in a primer/template system prone to primer dimer formation over a range of template concentrations. B. Corresponding standard curve comparing CleanAmp™ Turbo Primers and unmodified primers. PCR Conditions² on page 20.

CleanAmp™ Turbo Primers Outperform Other Fast Cycling Technologies

Next, CleanAmp™ Turbo Primers were compared with Full Velocity™ SYBR Green® QPCR Mastermix (Stratagene/Agilent), a commercially available kit which is formulated specifically for fast cycling PCR. The experiment involved amplification of a 245 bp target from Lambda genomic DNA. To accurately compare the two products in the same experiment, a 5 minute initial denaturation time necessary for the Full Velocity™ Mastermix was used with these cycling conditions, we found that Turbo Primers had slightly greater sensitivity than the Full Velocity™ Master Mix.

As depicted in Figure 11A, the NTC curve overlapped with the 5 copy sample with Full Velocity™ Master Mix. However, when Turbo Primers were employed, 5 copies of template amplified before the NTC curve. These findings are likely due to a lower level of off-target amplification by Turbo Primers. When the experimental results were plotted in a standard curve, both approaches displayed good linearity over the range of concentrations evaluated. Although the efficiencies were similar for each technology, reactions that employed Turbo Primers had lower Cq's. This allowed for even greater speed of amplicon detection in a fast cycling protocol.

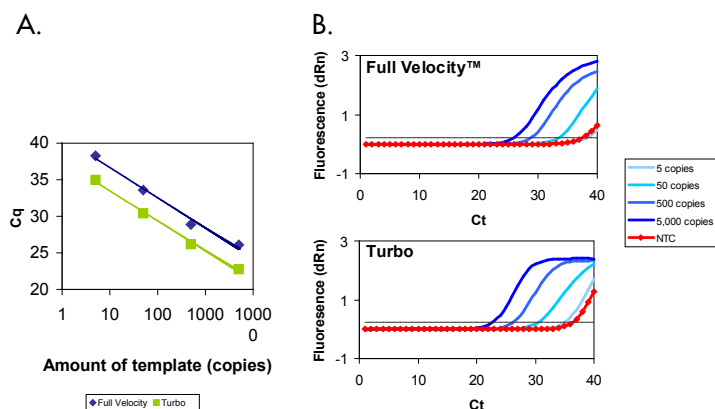


Figure 11: A. Amplification plots of CleanAmp™ Turbo Primers and Full Velocity™ in a primer/template system prone to primer dimer formation over a range of template concentrations. B. Corresponding standard curve comparing CleanAmp™ Turbo Primers and Full Velocity™ SYBR Green® QPCR Master Mix. PCR Conditions² on page 20.

Summary

In summation, CleanAmp™ Primers allow for greater control of primer hybridization and extension during PCR. Over a broad range of applications CleanAmp™ Primers reduce or eliminate off-target amplification. Greater amplicon yield is also achieved, due to improvement in specificity and sensitivity. It has been demonstrated that CleanAmp™ Primers outperform other technologies in multiple applications.

Which CleanAmp™ Primer is Best for My Application?

Turbo Primers	Precision Primers
Fast cycling	Standard cycling
Multiplex PCR	One-step reverse-transcription PCR
Improved amplicon yield	Improved specificity and limit of detection
Reduced mis-priming/primer dimer formation	Greatest reduction in mis-priming/primer dimer formation

PCR Conditions

1. PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂), Primers (0.5 μM), dNTPs (0.2 mM), 5 copies HIV-1 gDNA, Taq DNA polymerase (1.25 U), 50 μL. Thermal cycling conditions: 95°C (10 min); [95°C (40 sec), 56°C (30 sec), 72°C (1 min)]40X, 72°C (7 min).
2. PCR conditions (Turbo Primers): 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂), Primers (0.3 μM), 0.8 mM dNTPs, 0-10,000 copies Lambda gDNA, 3.75 U Taq DNA polymerase, 25 μL 1:2000 SYBR Green® I. PCR conditions (Unmodified Primers): 1X Full Velocity™ Master Mix (Stratagene/Agilent), Primers (0.15 μM). Thermal cycling conditions: 95°C (5 min)[95°C (10 sec), 66°C (30 sec)] 40X.
3. Thermal cycling conditions: 42°C (30 min), 95°C (10 min), [95°C (30 sec), 60°C (30 sec), 72°C (30 sec)] 30X, 72°C (5 min). Reaction conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂), Primers (0.5 μM), polydT primer (1 μM), 0.16 mM dNTPs, 0-0.25 μg Human brain total RNA, 25 U reverse transcriptase, 0.3 U Taq DNA polymerase, 25 μL.
4. PCR conditions (Turbo Primers): 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂), Primers (0.5 μM), polydT primer (1 μM), 0.16 mM dNTPs, 0-0.25 μg Human brain total RNA, 25 U reverse transcriptase, 0.3 U Taq DNA polymerase, 25 μL.

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Contact Information

For further information on CleanAmp™ Primers, please visit our website or contact TriLink directly:

CleanAmp™ Products online:

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