Application Forum

Improved PCR specificity with Hot Start PCR primers

Elena Hidalgo Ashrafi and Natasha Paul*,
TriLink BioTechnologies, Inc. San Diego, CA
*Corresponding author

Introduction

The polymerase chain reaction (PCR) is an indispensable DNA amplification technique that has been exploited in numerous areas, including molecular diagnostics. One major drawback of PCR is the competing amplification of undesired off-target products, which primarily occurs at ambient temperatures prior to PCR cycling (1). Hot Start PCR techniques aim to block the extension of primers in nonspecific complexes until higher, more stringent temperatures, thereby reducing off-target amplification. Herein, we describe a primer-based approach to Hot Start activation. CleanAmp™ Primers contain temperature-sensitive 4-oxo-tetradecyl (OXT) phosphotriester modifications which can be introduced at the 3′-terminal phosphodiester linkages of any primer (2,3). The OXT group blocks DNA polymerase primer extension at low-stringency conditions, but dissociates at the higher temperatures of PCR. This generates the unmodified primer, which can now be a substrate for the DNA polymerase. As a consequence, the primers are extended under higher-stringency conditions, diminishing the chance of amplifying off-target products.

There are two types of CleanAmp™ Primers, differing in the number of OXT groups that they contain. CleanAmp™ Turbo Primers contain only a single OXT modification at the 3′-terminal internucleotide linkage, while CleanAmp™ Precision Primers contain OXT modifications at the two 3′-terminal internucleotide linkages. The OXT modifications, which can be introduced using standard solid phase oligonucleotide synthesis, are removable at elevated temperatures to produce the corresponding unmodified primer (2,4). CleanAmp™ Turbo Primers are best suited for applications in which the primers need to be more readily available, such as fast cycling PCR and multiplex PCR. CleanAmp™ Precision Primers contain two modification groups and therefore require a slightly longer initial denaturation time to be activated (2). This slower-releasing characteristic provides an advantage in applications that require lower-temperature incubation, such as the reverse transcription (RT) step in a one-step RT-PCR protocol.

Material and methods

CleanAmp™ Primer synthesis:

CleanAmp™ Primers were synthesized by TriLink BioTechnologies, Inc. They were prepared using fast deprotecting phosphoramidites (Glen Research, Sterling, VA, USA) with OXT modifications introduced using CleanAmp™ Amidites (TriLink BioTechnologies, Inc. or Glen Research). After synthesis, CleanAmp™ Primers were isolated and purified using a solid phase extraction step (2) as described in further detail at trilinkbiotech.com/cleanamp/amidites.

Fast cycling qPCR:

A 365 bp target from HIV-1 genomic DNA was amplified using fast cycling PCR conditions. Reaction conditions included 1× PCR buffer [20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$; New England BioLabs, Ipswich, MA, USA], primers (1.5 µM; TriLink BioTechnologies, Inc; forward: 5′-GAATTGGGTGTCAACATAGCAGAAT, reverse: 5′-AATACTATGGTCCACACAACTATTGCT), dNTPs (0.8 mM; New England BioLabs), HIV gDNA (0-125 copies; Applied Biosystems, Foster City, CA, USA), SYBR Green™ I (0.15X; Invitrogen, Carlsbad, CA, USA), passive reference ROX dye (0.03 µM; Stratagene, La Jolla, CA, USA) and *Taq* DNA polymerase (3.75 U; Invitrogen) in a 25-µL reaction. Thermal cycling conditions were 40 cycles at 95°C for 10 sec and 66°C for 30 sec.

One-step RT-PCR:

Short fragments of the ABCC10 gene (61 bp; forward: 5′-GCGGGTTAAGCTTGTGACAGA, reverse: 5′-CCCACCCGCAGAACTTGA), ABCA5 gene (82 bp; forward: 5′-GGCTGCTATTCTGACCACTCACTATA, reverse: 5′-TTAACTGCCCAGACACCATGAT), the ABCA6 gene (114 bp; forward: 5′-CCATGAGAAATGTCCAGTTTCCT, reverse: 5′-TGCTGGGTTAAATTAGATATTGGTGTA), and the ABCA7 gene (143 bp; forward: 5′-TTTCTCTGGGACATGTGTAACTACTTG, reverse: 5′-TGTGATCGACCAGCCATACAG), were amplified from human trachea total RNA in a one-step RT-PCR protocol. Reaction set-ups included 1× PCR buffer [20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$ (Invitrogen)], PCR primers (0.5 µM, TriLink BioTechnologies, Inc.), oligo$(dT)_{18}$ primer (1 µM; TriLink BioTechnologies, Inc.), dNTPs (0.16 mM; New England BioLabs), human trachea total RNA (0.5 µg; Stratagene), M-MLV reverse transcriptase (50 U; Invitrogen), RNase Inhibitor (5U; New England BioLabs), and *Taq* DNA polymerase (0.6 U; Invitrogen) in a 50-µL reaction. Thermal cycling conditions utilized a reverse transcription step at 42°C for 30 min; 95°C for 10 min; followed by 45 cycles at 95°C for 15 sec, 60°C for 1 min, and a final extension step of 72°C for 5 min.

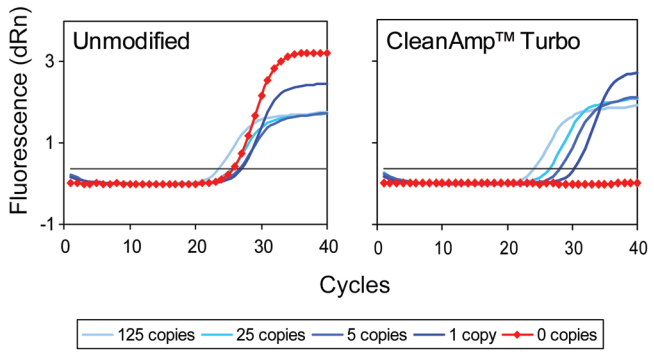

Figure 1. Comparison of CleanAmp™ Turbo and Unmodified PCR primers in fast cycling PCR, using real-time SYBR Green™ I detection.

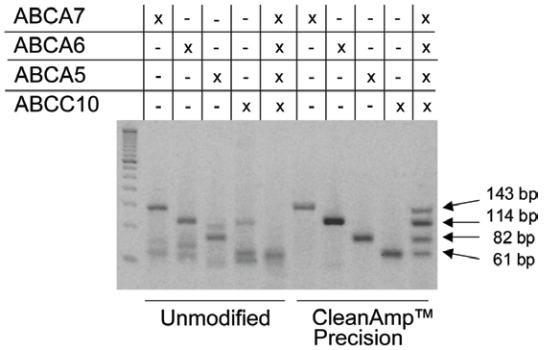

Figure 2. Comparison of CleanAmp™ Turbo and Unmodified PCR primers in one-step RT-PCR.

Results and discussion

Fast cycling protocols reduce the duration of PCR by combining annealing and extension steps. However, this reduction in assay time can also compromise sensitivity and specificity, especially since primer concentrations often need to be increased for optimal performance. To investigate the use of CleanAmp™ Turbo Primers in fast PCR, a 365 bp target, prone to primer dimer formation (1) was amplified in a qPCR experiment with SYBR Green™ I detection (Figure 1). Reactions employing CleanAmp™ Turbo Primers showed good sensitivity with robust amplification of input template amounts ranging 1–125 copies per reaction and no amplification in the absence of template (0 copies) (Figure 1). On the other hand, reactions with unmodified primers were plagued by primer dimer formation as evidenced by the early Cq (Quantification Cycle) in the amplification plots of the 0 copies reaction (Figure 1), which could be interpreted as a false positive result. Furthermore, when template was present, many of the reactions with unmodified primers had similar Cqs, providing little discrimination for detection of each of the template concentrations.

In further studies, CleanAmp™ Precision Primers and unmodified primers were compared for their ability to amplify four targets from human total RNA separately and simultaneously in one-step reverse transcription PCR (RT-PCR) (Figure 2). The implementation of multiplex one-step RT-PCR protocols provides a distinct advantage over a two-step reaction by substantially reducing the experimentation time and the number of handling steps. When each of the four targets were amplified individually using unmodified primers for the PCR step, each of the correct targets (61 bp, 82 bp, 114 bp, and 143 bp) were formed, but there was a significant amount of off-target products for each reaction (Figure 2). In comparison, reactions that employed CleanAmp™ Precision Primers for the PCR step displayed improved specificity and increased amplification yield (Figure 2). When all four targets were co-amplified in a single one-step RT-PCR set-up, reactions with unmodified PCR primers were unable to form all four targets, while setups with CleanAmp™ Precision PCR Primers efficiently formed each of the targets.

In summary, the choice of two CleanAmp™ Primer constructs, the single OXT-modified Turbo primers and the double OXT-modified Precision primers, allows the user to fine tune the kinetics of primer deprotection for the downstream application. For fast cycling PCR, CleanAmp™ Turbo Primers allow for high primer concentrations without compromised reaction specificity. CleanAmp™ Precision Primers minimize the need for extensive assay optimization for one-step RT-PCR, since the slower deprotection improves specificity by suppressing PCR primer extension during the less-stringent, lower-temperature RT step. These studies demonstrate that the substitution of CleanAmp™ Primers for unmodified primers provides improved performance for a number of PCR-based applications. CleanAmp™ Primers can be ordered through TriLink BioTechnologies or synthesized in-house. Learn how at trilinkbiotech.com/cleanamp.

References

1. Chou, Q., Russell, M., Birch, D.E., Raymond, J. and Bloch, W. (1992) Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. *Nucleic Acids Res*, **20**, 1717-1723.
2. Lebedev, A. (2009) Heat-activatable primers for Hot Start PCR: Oligonucleotide synthesis and basic PCR set-up. *Current Protocols in Nucleic Acid Chemistry*, (Accepted for publication).
3. Lebedev, A.V., Paul, N., Yee, J., Timoshchuk, V.A., Shum, J., Miyagi, K., Kellum, J., Hogrefe, R.I. and Zon, G. (2008) Hot start PCR with heat-activatable primers: a novel approach for improved PCR performance. *Nucleic Acids Res*, **36**, e131.
4. Ashrafi, E.H. and Paul, N. (2009) Heat-activatable primers for Hot Start PCR and Hot Start One-Step RT-PCR : End Point and Real Time experiments. *Current Protocols in Molecular Biology*, (Accepted for publication).

Contact

TriLink BioTechnologies, Inc., 9955 Mesa Rim Road
San Diego, CA 92121, Tel: (858) 546-0004 Fax: (858) 546-0020
info@trilinkbiotech.com, npaul@trilinkbiotech.com

Sponsored Paper. *BioTechniques* 47:XXX-XXX (September 2009)
doi 10.2144/000113240