Application Forum

Enhancing multiplex PCR efficiency using Hot Start dNTPs

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Introduction

Multiplex PCR is a widespread molecular biology technique for amplification of multiple targets in a single PCR experiment. This approach saves both time and money because several loci can be amplified simultaneously. Today, this technique is applied in numerous areas, including molecular diagnostics and forensics (1). Although its utility is unquestioned, multiplex PCR can be particularly difficult to optimize. Because a unique primer pair is included for each additional target, reactions become more prone to off-target amplifications, such as mis-priming and primer dimerization, than traditional PCR setups. These nonspecific amplifications can hinder the sensitivity and selectivity of multiplex PCR because key components in the reaction mix are consumed, resulting in diminished target amplicon yields. Furthermore, preferential amplification of certain targets in multiplex PCR experiments can result in an imbalance of target amplicon yield formation.

Many attempts have been made to improve the efficiency and specificity of multiplex PCR (2,3). Commonly used Hot Start approaches hold a key PCR reagent inactive until higher thermocycling temperatures are reached. Hot Start polymerases have shown tremendous promise in enhancing multiplex PCR, but

their use can significantly increase the cost of PCR experiments. An approach that has yet to be examined is the use of chemically modified dNTPs as a heat-activatable component in multiplex PCR (4). Because dNTPs are essential to PCR, modified components can easily take the place of standard dNTPs in the reaction mix without significantly altering existing protocols.

Hot Start dNTPs or CleanAmp™ dNTPs contain a thermolabile 3'-tetrahydrofuranyl (THF) protecting group that is released at high PCR temperatures (4,5). During the lower nonstringent temperatures of PCR setup, primers can interact nonspecifically and form mis-priming and primer dimerization products. The use of CleanAmp™ dNTPs blocks primer extension at these lower temperatures, thereby reducing off-target artifact accumulation during PCR. At Hot Start temperatures (~95°C), the 3'-THF protecting group is released, yielding a standard dNTP substrate suitable for DNA polymerase incorporation. Herein, we investigate the incorporation of CleanAmp™ dNTPs and how they can mitigate or eliminate the difficulties of multiplex PCR.

Materials and methods

Protocols for the duplex quantitative PCR experiment (Figure 1) consisted of 1× PCR buffer (20 mM Tris, pH 8.4, 50 mM KCl, 2.5 mM MgCl₂; Invitrogen), 2.5 U *Taq* DNA polymerase (Invitrogen), 0.2 μM primers (TriLink BioTechnologies), 0.4 mM

Figure 1. Comparison between standard and CleanAmp™ dNTPs for quantitative PCR detection of a 114- and a 185-bp target from mouse genomic DNA template. (A) Amplification plot for the 185-bp target. Resultant standard curves for detection of both targets in reactions employing (B) standard and (C) CleanAmp™ dNTPs.

Figure 2. Comparison between standard and CleanAmp™ dNTPs for amplification of multiple targets from mouse genomic DNA. Starting with a duplex reaction that amplified the 114- and 185-bp targets, primer pairs were sequentially added for the 214-, 293-, 388-, 515-, and 650-bp targets.

dNTPs (standard dNTPs, New England Biolabs; CleanAmp™ dNTPs, TriLink BioTechnologies), 0.03 μ M ROX reference dye, 0.1 μ M hydrolysis probe (114-bp target: 5' HEX-ACCAAGTGGGATTG-GCCAACTATGACTTCT-BHQ1 and 185-bp target: 5' FAM-TGCACT-TCAAGTCTCTGCTAAGCTAGGCTC-BHQ1), and template concentrations ranging from 0.05 to 500 ng of mouse genomic DNA (Promega). All 25- μ L reactions were set up in triplicate, with each reaction conducted in a single, thin-walled 200- μ L tube. The samples were run on a Stratagene MX3005P™ with the following thermal cycling conditions: 95°C (10 min); 35 cycles of 95°C (15 s), 60°C (30 s), and 72°C (1 min); and 72°C (5 min). The primer sequences employed in this study were specific for formation of a 114-bp (5' TGAGCCAGGAGAGCATCATTGAGG, 5' GACACCCAATTTCCACGCAGACAC) and a 185-bp (5' GCGGGTCTCCTTCTCCTTACTATCC, 5' CAGCTCAGTCTTCATGTGGTCTCC) amplicon.

For the experiments shown in Figure 2, reaction conditions were similar to those listed above, with the omission of reference dye and hydrolysis probe, and the inclusion of an additional 20 mM KCl and 50 ng mouse genomic DNA. In addition to the 114- and 185-bp primer pairs, primers specific for the formation of the 214-bp (5' TCGACTGCATGGTGTGAACGG, 5' TCCCTTATCAACTGCCCTAACTTCC), 293-bp (5' GAGGGTCGTGGGCGGTGTG, 5' TGGCTCTGGCTGGCTTGTATGG), 388-bp (5' CAGCACAGC-CACCACCAACG, 5' ATGGCACAGTCCTTCCCGATGG), 515-bp (5' AGGGAACCACAACCTAGCAAATACC, 5' ACACACCATGACCAAAGGCAACC), and 650-bp (5' AGAACAGCAGTACA-CAAGGCAAGC, 5' GGGAGTTCGCCAGTAAGCAAAGC) targets were employed. All samples were run in a BioRad thermal cycler using the thermal cycling protocol described above. After PCR, 20 μ L each sample was electrophoresed in a 2% agarose E-gel (Invitrogen) and visualized using an Alpha Innotech Corporation Multi Image Light Cabinet with CCD Camera.

Results and conclusion

While broadly used in a range of applications, multiplex PCR can be hampered by nonspecific amplicon artifacts that limit its overall specificity and efficiency. To investigate whether the use of CleanAmp™ dNTPs would improve the performance of a problematic duplex quantitative PCR experiment, reactions containing standard and CleanAmp™ dNTPs were compared (Figure 1). The amplification plot of the 185-bp target (Figure 1A) revealed that reactions using CleanAmp™ dNTPs had more robust log phases of amplification and increased sensitivity because the intended target was amplified across the entire input template range. However, reactions with unmodified dNTPs did not achieve the same level of performance. When the resultant Cq (quantification cycle) data was plotted in a standard curve, reactions employing standard dNTPs had varied results from replicate to replicate as evidenced by the large standard deviation on the graphs, especially at lower input template concentrations (Figure 1B). In contrast,

reactions employing the CleanAmp™ dNTPs showed very little deviation in the Cq values for each target across the entire template concentration range (Figure 1C). Overall, the use of CleanAmp™ dNTPs increased the limit of detection by an order of magnitude, increasing the sensitivity of the duplex reaction.

After conducting the duplex quantitative PCR experiments, the limits of CleanAmp™ dNTP utilization were investigated by performing experiments in which additional primer pairs were sequentially added until a seven-plex reaction was achieved (Figure 2). In all cases, the use of CleanAmp™ dNTPs improved the specificity and yield of amplicon formation while significantly reducing primer dimer formation. Moreover, PCR reactions with CleanAmp™ dNTPs amplified each of the seven targets with similar yields without any optimization of primer concentration. Most importantly, while PCR setups using standard dNTPs were ineffective, multiplex experiments employing CleanAmp™ dNTPs successfully amplified all targets of the seven-plex reaction.

Herein, we demonstrate the application of CleanAmp™ dNTPs for improved efficiency, sensitivity, and specificity in multiplex PCR. CleanAmp™ dNTPs are successful in amplifying a duplex reaction across a range of template concentrations, providing increased efficiency and sensitivity in multiplex PCR. In addition, the technology can also robustly amplify up to seven targets in a single reaction with better specificity than reactions with standard dNTPs. In conclusion, CleanAmp™ dNTPs provide an effective and cost-saving route to overcome the many challenges that traditionally plague multiplex PCR. Learn more at www.trilinkbiotech.com/cleanamp/dntps.

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