A GoTaq® PCR Primer: From Basic to RT-qPCR DNA Amplification

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Publication Date: 2010

Introduction

The polymerase chain reaction (PCR) is a relatively simple technique that amplifies a DNA template to produce specific DNA fragments in vitro. Amplification of DNA sequences by PCR requires only hours and requires very little biological starting material. Thus, PCR can achieve sensitive detection and high levels of amplification of specific sequences quickly. These features make the technique extremely useful, not only in basic research, but also in commercial uses, including genetic identity testing, forensics, industrial quality control and in vitro diagnostics. Basic PCR is commonplace in many molecular biology labs where it is used to amplify DNA fragments and detect DNA or RNA sequences within a cell or environment. However, PCR has evolved far beyond simple amplification and detection, and many extensions of the original PCR method have been described.

Basic PCR

The PCR process was originally developed to amplify short segments of a longer DNA molecule(1) . A typical amplification reaction includes target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. Once assembled, the reaction is placed in a thermal cycler and subjected to a series of different temperatures for set amounts of time. This series of temperature and time adjustments is referred to as one cycle of amplification. Each PCR cycle theoretically doubles the amount of targeted sequence (amplicon) in the reaction. Therefore, ten cycles should multiply the amplicon by a factor of about one thousand; 20 cycles, by a factor of more than a million in a matter of hours.

Each cycle of PCR includes steps for template denaturation, primer annealing and primer extension (Figure 1). The initial step denatures the target DNA by heating it to 94°C or higher. During denaturation, the two strands of DNA separate from one another, producing single-stranded DNA templates for replication. In the next step, the temperature is reduced to approximately 40–60°C, and the target-specific oligonucleotides anneal to the denatured template DNA, serving as primers for the DNA polymerase. Finally, the synthesis of new DNA begins as the reaction temperature is raised to the optimum for the DNA polymerase. The next cycle begins with a return to 94°C for denaturation.

Each step of the cycle should be optimized for each template and primer pair combination. If the temperature during the annealing and extension steps are similar, these two steps can be combined into a single step in which both primer annealing and extension take place. After 20–40 cycles, the amplified product may be analyzed for size, quantity, sequence, etc., or used in further experimental procedures.

Hot-Start PCR

Hot-start PCR is a common technique to reduce nonspecific amplification due to assembly of amplification reactions at room temperature. At these lower temperatures, PCR primers can anneal to template sequences that are not perfectly complementary. Since thermostable DNA polymerases have activity at these low temperatures (although in most cases the activity is less than 25%), the polymerase can extend misannealed primers. This newly synthesized region then acts as a template for primer extension and synthesis of undesired amplification products. However, if the reaction is heated to temperatures >60°C before polymerization begins, the stringency of primer annealing is increased, and synthesis of undesired PCR products is avoided or reduced.

Hot-start PCR also can reduce the amount of primer-dimer synthesized by increasing the stringency of primer annealing. At lower temperatures, PCR primers can anneal to each other via regions of complementarity, and the DNA polymerase can extend the annealed primers to produce primer dimer, which often appears as a diffuse band of approximately 50–100bp on an ethidium bromide-stained gel. The formation of nonspecific products and primer-dimers can compete for reagent availability with amplification of the desired product. Thus, hot-start PCR can improve the yield of specific PCR products.

To perform manual hot-start PCR, reactions are assembled on ice or at room temperature, but one critical component is omitted until the reaction is heated to 60–65°C, at which point the missing reagent is added. This omission prevents the polymerase from extending primers until the critical component is added at the higher temperature where primer annealing is more stringent. However, this method is tedious and increases the risk of contamination.

A second, less labor-intensive approach involves the reversible inactivation or physical separation of one or more critical components in the reaction. For example, the magnesium or DNA polymerase can be sequestered in a wax bead, which melts as the reaction is heated to 94°C during the denaturation step, releasing the component only at higher temperatures(2) (3) (4) . The DNA polymerase also can be kept in an inactive state by binding to an oligonucleotide, also known as an aptamer(5) (6) or an antibody(7) (8) . This bond is disrupted at the higher temperatures, releasing the functional DNA polymerase. Finally, the DNA polymerase can be maintained in an inactive state through chemical modification(9) .

Reverse Transcriptase (RT) PCR

Thermostable DNA polymerases used for basic PCR require a DNA template, and as such, the technique is limited to the analysis of DNA samples. Yet numerous instances exist in which amplification of RNA would be preferred. To apply PCR to the study of RNA, the RNA sample must first be reverse transcribed to cDNA to provide the necessary DNA template for the thermostable polymerase. This process is called reverse transcription (RT), hence the name RT-PCR. After the initial reverse transcription step to produce the cDNA template, basic PCR is carried out to amplify the target sequence.

The quality and purity of the RNA template is crucial to the success of RT-PCR. Total RNA or poly(A)+ RNA can be used as the starting template, but both must be intact and free of contaminating genomic DNA. Specific capture of poly(A)+ RNA will enrich a targeted message so that less of the reverse transcription reaction is needed for subsequent amplification. The efficiency of the first-strand synthesis reaction, which can be related to the RNA quality, also will significantly affect amplification results.

Quantitative Endpoint PCR

PCR and RT-PCR are generally used in a qualitative format to evaluate biological samples. However, a wide variety of applications, such as determining viral load, measuring responses to therapeutic agents and characterizing gene expression,

would be improved by quantitative determination of target abundance. Theoretically, this should be easy to achieve, given the exponential nature of PCR, because a linear relationship exists between the number of amplification cycles and the logarithm of the number of molecules. In practice, however, amplification efficiency is decreased because of contaminants (inhibitors), competitive reactions, substrate exhaustion, polymerase inactivation and target reannealing. As the number of cycles increases, the amplification efficiency decreases, eventually resulting in a plateau effect.

Normally, quantitative PCR requires that measurements be taken before the plateau phase so that the relationship between the number of cycles and molecules is relatively linear. This point must be determined empirically for different reactions because of the numerous factors that can affect amplification efficiency. Because the measurement is taken prior to the reaction plateau, quantitative PCR uses fewer amplification cycles than basic PCR. This can cause problems in detecting the final product because there is less product to detect.

To monitor amplification efficiency, many applications are designed to include an internal standard in the PCR. One such approach includes a second primer pair that is specific for a “housekeeping” gene (i.e., a gene that has constant expression levels among the samples compared) in the reaction(10) (11) . Amplification of housekeeping genes verifies that the target nucleic acid and reaction components were of acceptable quality but does not account for differences in amplification efficiencies due to differences in product size or primer annealing efficiency between the internal standard and target being quantified.

The concept of competitive PCR—a variation of quantitative PCR—is a response to this limitation. In competitive PCR, a known amount of a control template is added to the reaction. This template is amplified using the same primer pair as the experimental target molecule but yields a distinguishable product (e.g., different size, restriction digest pattern, etc.). The amounts of control and test product are compared after amplification. While these approaches control for the quality of the target nucleic acid, buffer components and primer annealing efficiencies, they have their own limitations(12) (13) , including the fact that many depend on final analysis by electrophoresis.

Numerous fluorescent and solid-phase assays exist to measure the amount of amplification product generated in each reaction, but they often fail to discriminate amplified DNA of interest from nonspecific amplification products. Some of these analyses rely on blotting techniques, which introduce another variable due to nucleic acid transfer efficiencies, while other assays were developed to eliminate the need for gel electrophoresis yet provide the requisite specificity. Real-time PCR, which provides the ability to view the results of each amplification cycle, is a popular way of overcoming the need for analysis by electrophoresis.

Quantitative Real-Time PCR

The use of fluorescent DNA-binding dyes or fluorescently labeled oligonucleotide probes or primers to detect and quantitate a PCR product allows quantitative PCR (qPCR) to be performed in real time. Specially designed instruments perform both thermal cycling to amplify the target and fluorescence detection to monitor PCR product accumulation. DNA-binding dyes are easy to use but do not differentiate between specific and nonspecific PCR products and are not conducive to multiplex reactions. Fluorescently labeled nucleic acid probes have the advantage that they react with only specific PCR products, but they can be expensive and difficult to design. Some qPCR technologies employ fluorescently labeled PCR primers instead of probes. One example is the Plexor® technology, which requires only a single fluorescently labeled primer, and is compatible with multiplex PCR and allows specific and nonspecific amplification products to be differentiated(14) (15) .

The use of fluorescent DNA-binding dyes, like Promega BRYT™ Green dye, is one of the easiest qPCR approaches. The dye is simply added to the reaction, and fluorescence is measured at each PCR cycle. Because fluorescence of these dyes increases dramatically in the presence of double-stranded DNA, DNA synthesis can be monitored as an increase in

fluorescent signal. However, preliminary work often must be done to ensure that the PCR conditions yield only specific product. In subsequent reactions, specific amplification can be verified by a melt curve analysis. Thermal melt curves are generated by allowing all product to form double-stranded DNA at a lower temperature (approximately 60°C) and slowly ramping the temperature to denaturing levels (approximately 95°C). The product length and sequence affect melting temperature (T_m), so the melt curve is used to characterize amplicon homogeneity. Nonspecific amplification can be identified by broad peaks in the melt curve or peaks with unexpected T_m values. By distinguishing between specific and nonspecific amplification products, the melt curve adds a quality control aspect during routine use. The generation of melt curves is not possible with assays that rely on the 5′→3′ exonuclease activity of *Taq* DNA polymerase, such as the probe-based TaqMan® technology.

The GoTaq® Family of Products: Basic to Quantitative PCR Applications

Set up your **basic PCR** amplifications in less than a minute using the **GoTaq® Master Mixes**. Each mix is supplied with enzyme, magnesium, dNTPs and buffer in one tube; you add template, water and primers. You can choose from green or colorless reaction buffers that allow you to go directly from thermal cycler to gel analysis. These buffers contain a compound that increases sample density, so that samples sink easily into the wells of an agarose gel. The green buffer also contains two dyes (yellow and blue) that separate to allow easy monitoring during electrophoresis. The blue dye comigrates at the same rate as 3–5kb DNA fragments in a 1% agarose gel. The yellow dye migrates ahead of primers (<50bp). Use the green reaction buffer for direct-to-gel analysis after amplification and the colorless reaction buffer for post-amplification analysis by fluorescence or absorbance without prior purification of the DNA. Or, if your experimental system requires the flexibility of separate components, choose one of the **GoTaq® PCR Core Systems**, or order the buffer and **GoTaq® DNA Polymerase** as standalone items.

GoTaq® Hot Start Polymerase contains high-performance GoTaq® DNA Polymerase bound to a proprietary antibody that blocks polymerase activity. Polymerase activity is eliminated or minimized at temperatures below 70°C and restored during the initial denaturation step, increasing reaction specificity. The GoTaq® Hot Start Polymerase also is available in **GoTaq® Hot Start Master Mixes (Green or Colorless)**, premixed, ready-to-use solutions containing GoTaq® Hot Start Polymerase, magnesium, dNTPs and buffer. You simply add your template, water and primers.

The **GoTaq® qPCR Master Mix** is a qPCR reagent system that contains BRYT™ GREEN, a proprietary fluorescent DNA-binding dye that often exhibits greater fluorescence enhancement upon binding to double-stranded DNA and less PCR inhibition than the commonly used SYBR® Green I dye. The dye in the GoTaq® qPCR Master Mix enables efficient amplification, resulting in earlier quantification cycle (C_q, formerly known as cycle threshold [C_t]) values and an expanded linear range using the same filters and settings as SYBR® Green I or FAM™ Dye. The GoTaq® qPCR Master Mix is provided as a simple-to-use, stabilized 2X formulation that includes all components for qPCR except sample DNA, primers and water.

GoTaq® 2-Step RT-qPCR System is a reagent system for quantitative analysis of RNA using a two-step reverse transcription-quantitative PCR (RT-qPCR) protocol. The components and protocol allow sensitive and linear two-step RT-qPCR quantitation over a wide range of RNA templates using the GoScript™ Reverse Transcription System for cDNA synthesis followed by the GoTaq® qPCR Master Mix. The GoTaq® 2-Step RT-qPCR System contains a new fluorescent DNA-binding dye, BRYT™ GREEN dye, that often exhibits greater fluorescence enhancement upon binding to double-stranded DNA (dsDNA) than SYBR® Green I. GoTaq® qPCR Master Mix can be used with any real-time instrument capable of detecting SYBR® Green I or FAM™ dye.

No matter what your PCR application or need, there is a GoTaq® System or product that can help you achieve your research goals. Use the amplification product selector to find the right product for your experimental needs.

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