FAQs: PCR Polymerases from Takara Bio

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PCR Basics

Q1 What parameters do I need to consider when designing primers?

Answer 1. Consider the following issues when designing primers:

In general, use 20- to 25-mer primers. It is important to design primers with melting temperature (Tm)[1] values that are compatible with the PCR conditions that will be used. For more specific details, please refer to the user manual for each product.

FAQs: PCR Polymerases, cont.

When amplifying long DNA (≥10 kb), 25- to 35-mer primers may provide better results. We also recommend using primer-design software[2] to design primers that have Tm values of 65°C or above.

It is possible to increase amplification specificity by using primers that have an overall GC content between 50% and 60% and have lower GC content of the final 10 bases at the 3′ end . Furthermore, to prevent mis-priming, we recommend avoiding T residue at the 3′ terminus.

To ensure that the forward and reverse primers cannot anneal to each other, avoid complementary sequences of three bases or more at the 3′ termini of the primers. Also, the forward and reverse primers should have Tm values that are as similar as possible.

To prevent the formation of unwanted secondary structures, ensure that there are no self-complimentary sequences within individual primers.

For GC-rich target sequences, primers with Tm values greater than 60°C are recommended.

When performing multiple PCR reactions using different primer pairs and using identical thermal cycling conditions, we recommend that each primer be designed to have a Tm value that is appropriate for the PCR enzyme and conditions used.

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[1]For calculating Tm: Tm (°C) = 2 (nA + nT) + 4 (nC + nG) – 5
This formula applies to primers no longer than 25 bases. For longer primers, we recommend using primer design software to determine the Tm value.

[2] Recommended primer design software: Primer3 (Steve Rozen and Helen J. Skaletsky (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386); OLIGO Primer Analysis Software (Molecular Biology Insights Inc.)

Q2 What is the optimum amount of template to use?

Answer 2. The amount of template necessary for PCR depends on the template type (i.e., genomic DNA, plasmid DNA, or reverse transcription product).

For PrimeSTAR® HS DNA Polymerase (Cat.# R010A), excessive template tends to inhibit amplifications. In contrast, PrimeSTAR Max DNA Polymerase (Cat.# R045A) tolerates a broader range of template quantity. In general, reaction conditions may be adjusted depending on the type, quantity, and quality of template.

The following are the standard recommended amounts of template per 50 µl reaction:

- Human genomic DNA: 5 to 200 ng
- *E. coli* genomic DNA: 100 pg to 100 ng
- cDNA library: 1 to 200 ng
- λ DNA: 10 pg to 10 ng
- Plasmid DNA: 10 pg to 1 ng

When using 25- to 30-mer primers, two-step PCR usually provides good results.

Q3 Which conditions are particularly important to consider when planning your PCR?

Answer 3. Please consider the following parameters:

- *Preheating before PCR cycling (initial denaturation)*

  PCR enzymes offered by Takara Bio do not require preheating for enzyme activation. For the preheating step that is

sometimes required to denature complex templates, such as genomic DNA, 94°C for 1 min. is sufficient. Excessive heat treatment may lead to enzyme inactivation.

Terra PCR Direct Polymerase Mix from Clontech, which is used for direct PCR amplification from tissue without need for DNA extraction and purification, requires preheating at 98°C for 2 min.

- *Denaturing conditions*

  Denaturing conditions should be selected by considering the thermal cycler model and the type of tube that will be used. A general guideline is to denature at 94°C for 10–30 sec. or at 98°C for 1–10 sec.

  If using a more heat-resistant enzyme such as one of the PrimeSTAR polymerases, we recommend using a high temperature and short duration (i.e., at 98°C for 5–10 sec.) for the denaturation step.

- *Annealing conditions*

  Amplification efficiencies and specificities vary depending on the annealing conditions. Using annealing temperatures that are too low may result in mis-priming and nonspecific amplification, thereby leading to low yields of the desired product. Therefore, amplification efficiency and specificity can be improved by adjusting the annealing temperature according to the primer's Tm or by performing two-step PCR.

  – For *Taq* enzymes, the recommended annealing time is 30 sec.

  – Enzymes in the PrimeSTAR series have excellent priming efficiencies. Therefore, it is important to use a short annealing time of 5–15 sec. Excessively long annealing times may lead to mispriming-induced nonspecific amplification products or smears.

  – When amplifying short sequences of no more than 1 kb, a three-step PCR strategy is recommended. For GC-rich targets or amplifications of long sequences (>10 kb), a two-step PCR strategy is recommended.

- *Extension time*

  In general, an extension time of 1 min/kb is recommended.

  When using high-speed SpeedSTAR™ HS DNA Polymerase (Cat.# RR070A) or SapphireAmp® Fast PCR Master Mix (Cat.# RR350A), use a reaction rate of 10 sec per kb of amplified product (e.g., 10 sec. for a 1 kb product, 20 sec. for a 2-kb product, etc.).

  PrimeSTAR Max DNA Polymerase (Cat.# R045A) and PrimeSTAR GXL DNA Polymerase (Cat.# R050A), both of which contain Takara Bio's proprietary elongation factor, allow for high-speed reactions at 5–20 sec/kb. If performing PCR with PrimeSTAR Max or PrimeSTAR GXL polymerase in the presence of excess template, set the elongation time to 1 min/kb.

Recommended Enzymes

Q4 Which enzymes provide superior fidelity?

Answer 4. All of Takara Bio's PrimeSTAR series enzymes provide better fidelity than that of *Pfu* DNA polymerase, a conventional benchmark high-fidelity PCR enzyme and Phusion® enzymes. PrimeSTAR Max DNA Polymerase (Cat.# R045A) provides the highest fidelity. When this enzyme was used to amplify the entire pUC119 plasmid, sequence analysis detected only four mutations out of 370,656 total bases sequenced (an error rate of 0.00108%).

In addition, compared to other enzymes, PrimeSTAR Max DNA Polymerase replicates repeated sequences with markedly better fidelity and exhibits a lower rate of template-exchange (formation of chimeric molecules) for analogous sequences.

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Q5 Which enzyme is optimal for amplifying GC-rich target sequences?

Answer 5. Consider the following enzymes:

- **First Choice:**

  PrimeSTAR GXL DNA Polymerase (Cat.# R050A) is effective for target sequences that are GC-rich such as bacterial genomic DNA. This enzyme has been used successfully to amplify a region with about 70% GC content in a standard reaction using the buffer provided with the enzyme. PrimeSTAR GXL polymerase is the most effective enzyme for GC-rich templates. It facilitates high-fidelity amplifications with very few errors.

- **Second Choice:**

  For GC-rich targets that have rigid structures and are difficult to amplify with PrimeSTAR GXL, use *TaKaRa LA Taq*® with GC Buffer (Cat.# RR02AG). Try GC Buffer I first. GC Buffer I facilitates the amplification of long products. Buffer II is effective for target templates with complex and rigid higher-order structure, although it is optimally used when amplifying shorter products.

Q6 Which enzymes are suitable for long range PCR?

Answer 6: PrimeSTAR GXL DNA Polymerase (Cat.# R050A) is recommended for accurate amplification of products greater than 6 kb. Amplification of 30 kb products has also been accomplished with this enzyme using human genomic DNA as template. As a secondary choice, we recommend *TaKaRa LA Taq* DNA Polymerase (Cat.# RR002A**).**

Q7 Which enzymes are suitable for fast PCR?

Answer 7: For fast PCR applications, we recommend PrimeSTAR Max polymerase for highest fidelity (Cat.# R045A), SapphireAmp Fast PCR Master Mix (Cat.# RR350A) for streamlined workflow and fast colony PCR, and SpeedSTAR HS DNA Polymerase (Cat.# RR070A) for SNP genotyping and fast long range PCR.

- PrimeSTAR Max contains Takara Bio's proprietary elongation factor and exhibits excellent priming efficiency. With extension times as short as 5 sec/kb and an annealing time of only 5 seconds, PrimeSTAR Max offers some of the fastest reaction speeds available − as well as exceptionally high fidelity. This enzyme is recommended for cloning and expression studies.
- SapphireAmp Fast PCR Master Mix is an economical choice for high-throughput projects. This enzyme is formulated to include high speed polymerase, optimized buffer, dNTP mixture, gel loading dye (blue), and a density reagent in a 2X premix. Since it requires an extension time of only 10 sec/kb, colony PCR reactions can be completed in less than 1 hour for inserts up to 1 kb.
- SpeedSTAR HS is highly efficient and can reliably perform PCR amplifications with extension times of 10−20 sec/kb.

Q8 Which enzymes are optimal for amplifying AT-rich target sequences?

Answer 8: *TaKaRa Ex Taq*® DNA Polymerase (Cat.# RR001A) and PrimeSTAR® GXL DNA Polymerase (Cat.# R050A) are effective for AT-rich target sequences such as genomic DNA containing introns or AT-rich regions of mitochondrial DNA. We recommend PrimeSTAR GXL polymerase for AT-rich amplifications that require high accuracy. Using the standard PCR protocol and the reaction buffer provided, PrimeSTAR GXL polymerase can amplify targets containing AT-rich regions and targets with >60% AT content, whereas these results cannot be achieved with most other PCR enzymes. PrimeSTAR GXL polymerase is the most potent enzyme in the PrimeSTAR enzyme series for use with AT-rich templates. However, this enzyme cannot be used to amplify bisulfite-treated DNAs and other uracil-containing templates.

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Q9 Which enzymes can be used to amplify bisulfite-treated DNAs?

Answer 9: Several choices are available:

- EpiTaq™ HS (for bisulfite-treated DNA) (Cat.# R110A) is optimized for PCR amplifications using bisulfite-treated DNA containing uracil as template. Designed for PCR for COBRA/Bisulfite sequencing analyses, it is well-suited for methylation analysis of CpG islands and also for DNA amplification with AT-rich and GC-rich templates.
- The EpiScope® MSP Kit (Cat.# R100A) is designed specifically for methylation-specific PCR (MSP) assays.
- Alternatively, *TaKaRa Taq*® (Cat.# R001A) and *TaKaRa Taq* Hot Start Version (Cat.# R007A), both of which lack 3→5′ exonuclease activity, may be used in some instances.

Q10 Which enzymes are better at tolerating the presence of PCR -inhibiting agents?

Answer 10: *TaKaRa Ex Taq*® DNA Polymerase (Cat.# RR001A) is exceptionally robust, even in the presence of PCR inhibitors such as polyphenols found in crude DNA extracts from plant tissue. PrimeSTAR GXL DNA Polymerase (Cat.# R050A) is recommended when high fidelity is required. Although PrimeSTAR GXL polymerase generally produces satisfactory results with the standard protocol, doubling the enzyme concentration may improve results if high concentrations of inhibitors are present.

Alternatively, Terra™ PCR Direct Polymerase from Clontech allows direct amplification from crude samples that contain high levels of PCR inhibitors and that are difficult to amplify by standard PCR enzymes. Terra PCR Direct Polymerase can efficiently amplify a wide range of targets, including GC- or AT-rich targets. This enzyme can also be used for direct PCR of blood samples.

However, if amplification products still cannot be produced, it may be necessary to purify the template DNA.

Q11 Which enzymes are suitable for amplifying DNA prepared from paraffin sections?

Answer 11: We recommend PrimeSTAR GXL DNA Polymerase (Cat.# R050A) for amplifying DNA isolated from paraffin sections. In addition, Terra™ Direct FFPE Kit from Clontech (Cat.# 639284) is available for crude DNA preparation and direct PCR amplification from paraffin-embedded tissue sections.

If it is necessary to extract DNA from paraffin-embedded tissue, TaKaRa DEXPAT™ Easy (Cat.# 9104) and TaKaRa DEXPAT Reagent (Cat.# 9091) enable quick, efficient DNA preparation for PCR, even from slides or samples stored for years.

Q12 Which enzymes faciliate efficient workflow when analyzing multiple samples by gel electrophoresis after PCR (e.g., genotyping screens)?

Answer 12: We recommend EmeraldAmp® GT PCR Master Mix (Cat.# RR310A). This product is completely premixed, making it easy to prepare PCR reaction mixtures which can be loaded directly on an agarose gel for electrophoresis after the reaction. For an efficient workflow with minimal pipetting, EmeraldAmp GT PCR Master Mix contains a green tracking dye. Additionally, density agent is included to aid in gel loading.

EmeraldAmp PCR Master Mix can amplify targets up to 10 kb in length, including targets that are GC- or AT-rich. Additionally, PCR products generated with EmeraldAmp GT PCR Master Mix can be used directly for restriction enzyme digestion, sequencing, or TA-cloning without need for further purification.

Q13 Which enzymes are suitable for colony PCR?

Answer 13: We recommend SapphireAmp Fast PCR Master Mix (Cat.# RR350A) and EmeraldAmp PCR Master Mix (Cat.# RR300A) for colony PCR. These enzyme preparations tolerate the presence of substantial bacterial nucleic acid carry-over. Because tracking dye and density agent are included in these master mixes, the PCR reaction mixtures may be loaded directly on an agarose gel for electrophoresis.

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Q14 What precautions should be taken when using an inosine-containing primer?

Answer 14: *TaKaRa Taq* (Cat.# R001A) and *TaKaRa Taq* Hot Start Version (Cat.# R007A) enzymes are compatible with inosine-containing primers.

It is important to note that inosine-containing primers should **not** be used with PCR enzymes that have 3'→5' exonuclease activity (e.g., PrimeSTAR HS DNA Polymerase, PrimeSTAR Max DNA Polymerase, PrimeSTAR GXL DNA Polymerase, *TaKaRa Ex Taq*, or *TaKaRa LA Taq*) or Terra Direct PCR Polymerase. When using one of these PCR enzymes, we recommend using mixtures of degenerate primers with A, T, G, and C at the desired position(s) rather than inosine-containing primers when performing degenerate PCR .

Q15 What is the terminal structure of each enzyme's amplification product? What are the most suitable cloning methods?

Answer 15: Due to the nature of PCR product termini, we recommend using the following cloning methods:

- **For enzymes in the PrimeSTAR series:**

  These enzymes exhibit substantial 3→5' exonuclease activity and primarily generate amplification products with blunt ends. Therefore, we recommend using the Mighty Cloning Reagent Set (Blunt End) (Cat.# 6027) for blunt-end cloning.

- **For *Taq* series and Terra enzymes:**

  TaKaRa Taq, *TaKaRa Ex Taq*, *TaKaRa LA Taq*, SpeedSTAR HS, EmeraldAmp, SapphireAmp Fast, and Terra enzymes primarily yield amplification products containing 3'-dA overhangs, which facilitates TA cloning. Cloning in blunt-end vectors is also possible by using the Mighty Cloning Reagent Set (Blunt End) (Cat.# 6027).

Troubleshooting

Q16 If no amplification products are obtained, which parameters should be considered first?

Answer 16. For all enzymes:

- Ensure that the primer sequences, lengths, and GC-content are appropriate. Primers shorter than 20 bases may be difficult to use for amplification with two-step PCR.
- Amplification may not be successful for forward and reverse primers whose Tm values differ by 5°C or more. Using an annealing temperature based on the lower Tm value of the two primers may improve results.
- Avoid using templates of low purity and templates that contain PCR inhibitors.
- Use an appropriate volume for the reaction mixture. Depending on the thermal cycler and PCR tube used, small reaction volumes (≤10 µl) may not amplify well.

For PrimeSTAR HS DNA Polymerase:

- Use an appropriate amount of template. If the template is human genomic DNA or a cDNA library, try using no more than ~100 ng of template for a 50 µl reaction mixture.
- Try extension times of at least 1 min/kb.
- Increasing the concentration of primers may improve results.

For PrimeSTAR Max DNA Polymerase:

- The extension time may need to be adjusted if the reaction mixture contains excess template. If the amount of template exceeds 200 ng in a 50 µl reaction mixture, set the extension time between 30 sec/kb and 1 min/kb.

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- Increasing the concentration of primers may improve results.

For SpeedSTAR HS DNA Polymerase:

- Although the standard extension time is 10 sec/kb, try increasing to ~0.5 min/kb for complex templates such as human genomic DNA.

Q17 If nonspecific amplification bands or smears are obtained, what can be done to improve the results?

Answer 17: For all Takara Bio enzymes:

- Use primers with higher specificity.
- Confirm that primers, enzyme, and template are present in the appropriate amounts and make sure that the extension time is not excessively long.
- Try increasing the annealing temperature by increments of 2°C, or change to a two-step PCR protocol.
- When the template contains small amounts of a target DNA, nested PCR may be effective.

For PrimeSTAR HS and PrimeSTAR Max DNA Polymerases:

- Check the annealing time setting for three-step PCRs. To achieve specific amplifications, it is essential to use a short annealing time (5 or 15 sec).

For PrimeSTAR GXL DNA Polymerase:

- To amplify targets less than 1 kb in length, design primers with Tm values higher than 55°C, and use an annealing temperature of 60°C. If the primer Tm values are 55°C or lower, try shorter extension times of between 5 and 10 sec/kb.

For *TaKaRa Ex Taq* and *TaKaRa LA Taq* DNA Polymerases:

- The Hot Start versions of these enzymes may provide improved results for some primers.

For SpeedSTAR HS DNA Polymerase:

- Excessively long extension times may result in smearing. The general recommendation for extension times for this enzyme is 10 to 20 sec/kb. If PCR yield is low, try increasing the number of cycles by 5.